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THE INFECTED CELL COUNT METHOD OF TITRATION OF FELINE PNEUMONITIS VIRUS

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Advances in the field of metazoan cell cultivation of the past few years[1] have opened a new field of methodology in animal virus infection. While exploiting this new field, Dulbecco[2] developed a quantitative virus technique using the virus of Western equine encephalomyelitis. When layered on a sheet of chick embryo connective tissue cells in appropriate dilutions, this virus produced areas of cytolysis analogous to the bacteriophage "plaques."

Dulbecco's method is applicable to viruses which readily multiply in connective tissue cells of the chick embryo and are highly destructive of their host cells. It can be profitably applied to the study of many problems of viral infection, growth and genetics. Additional methods, however, are required for the study of representatives of those viruses which have greater affinity for cells of ectodermal or entodermal derivation and which do not necessarily destroy their host cells.

Formation of inclusion bodies is an important characteristic of many viruses. It can be used for the development of a quantitative assay method provided that single layers of host cells can be obtained for microscopic examination and a constant association between inclusion body formation and infection can be established.

This paper describes the development of a quantitative assay method based on counting cells containing viral inclusions. It can be applied to some viruses which cannot be studied by Dulbecco's method, and may possibly yield information of a different nature. The virus selected for the development of this method is the agent of feline pneumonitis, which has proven in many previous investigations[3–7] to be an excellent test organism. Since the agent of feline pneumonitis had not previously been cultivated in explanted tissues, several difficulties had to be surmounted before a quantitative method could be developed. The main efforts were directed towards the following problems: (a) surveying methods of cultivation of the entodermal cells of the yolk sac, which proved to be the most susceptible to feline pneumonitis infection; (b) studying the morphology and development of feline pneumonitis virus in tissue culture and its effect on host cells; (c) developing a method of counting inclusion bodies; and (d) studying the factors

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1. Pomerat, C. M. 1951, Methods in Medical Research, **4**: 198–291.
2. Dulbecco, R. 1952, Proc. Nat. Acad. Sc. **38**: 747–752.
3. Weiss, E. 1949, J. Infect. Dis. **84**: 124–149.
4. Weiss, E. 1950, J. Infect. Dis. **87**: 249–263.
5. Gogolak, F. M. and Weiss, E. 1950, J. Infect. Dis. **87**: 264–274.
6. Moulder, J. W. and Weiss, E. 1951, J. Infect. Dis. **88**: 56–67.
7. Moulder, J. W. and Weiss, E. 1951, J. Infect. Dis. **88**: 68–76.

which most strongly influence infectivity.‡

The infected cell count method has been proven satisfactory in a preliminary study of the kinetics of feline pneumonitis virus infection. Further studies are required to adequately control the many factors which affect its sensitivity and precision.

MATERIALS AND METHODS

Viruses.—The strain of feline pneumonitis virus used in these experiments was first isolated by Baker[8] and has been maintained in the laboratory by yearly or biyearly passage in the yolk sac of chick embryos and by storage at −75 to −55 C. About 5 yolk sac passages have intervened since the study by Moulder and Weiss.[6] The strain of murine pneumonitis was first isolated by Nigg and Eaton[9] and obtained from the Viral and Rickettsial Registry of the American Type Culture Collection. Pools of virus were prepared from infected yolk sacs shaken for one-half hour in twice their volume of balanced salt solution. The supernatant fluid was stored in 0.5 ml amounts in an electric freezer at −60 C.

Chick embryos.—It was not possible to secure eggs from the same source throughout the course of this work. At first, fertile eggs of white Rock hens were used. But, since the diet of these hens contained a heavy concentration of antibiotics, growth of the virus was affected, and eggs of a different source had to be used. For a short time eggs of White Leghorn hens were available, but the last part of this work was carried out with those of the Nichols strain of the New Hampshire type. Although, as explained in a later section, individual embryos varied in susceptibility to virus infection, no obvious differences were encountered in the characteristics of the three strains. The fertile eggs were incubated in a rotating incubator at 37.5 C until used for explantation, yolk sac inoculation, or preparation of embryo extract.

Tissue culture reagents.—Hanks' balanced salt solution (BSS)[10] was used as diluent. It was prepared in 10-fold strength, stored in the refrigerator with chloroform as a preservative, and diluted for the needs of 1 or 2 weeks. After sterilization by autoclaving, enough 1.4% sodium bicarbonate was added aseptically to bring the pH of the solution to 7.6.

Chicken serum (CS) was obtained by heart puncture of young White Rock roosters kept on an antibiotic-free diet. The blood was allowed to clot by incubation at 37 C for 1 hour. Syneresis was stimulated by rapidly cooling the blood to 4 C after the period of incubation. Chicken plasma (CP) was also obtained by heart puncture. The blood was rapidly mixed with heparin to a final concentration of 1:50,000, cooled in iced water and centrifuged twice at 3500 r.p.m. for 10 to 20 minutes. The plasma was distributed in appropriate amounts in Wassermann tubes and stored at −60 C.

Embryo extract (EE) was prepared from 9- to 10-day-old chick embryos. About 6 to 10 embryos were placed in a glass tissue homogenizer and an equal volume of BSS added. The tissues were homogenized and maintained at 6 C for 1 or 2 hours, with occasional shaking. After centrifugation at 3000 r.p.m. for 20 minutes, the supernatants were dispensed in Wasserman tubes and stored at −60 C.

Bovine serum ultrafiltrate (UF), Fischer V-614 (V614) medium, and perforated cellophane were obtained from Microbiological Associates, Inc. Bovine plasma albumin, 35% solution (PA), was obtained from Armour Laboratories.

Tissue culture procedures.—The procedures varied considerably throughout the investigation, details of which are mentioned in other sections of the paper. In general, they were modifications of the following method: The embryo was poured into a Petri plate and the area to be explanted was transferred to another Petri plate. After several washings in BSS, the tissue was cut into explants, 1 to 2 mm in diameter, and washed until no yolk could be detected in the supernatant fluid. The explants were then placed on 11×22 mm coverslips or roller tubes and embedded in plasma, to which a small amount of embryo extract was added. The coverslips were placed in Porter flasks as soon as coagulation had occurred and nutrient fluid was added to the cultures. Some of the Porter flasks and roller tubes were rotated in a commercial tissue roller apparatus (Wyble Co.) at 12 rotations per minute. In other cases the flasks were placed on a shaker (Day Co.) which moved back and forth 54 times a min-

‡ Portions of this work were presented May 1, 1952, at the General Meeting of the Society of American Bacteriologists in Boston, and October 17, 1952, at the joint Meeting of the Indiana Academy of Science and the Indiana Branch of the Society of American Bacteriologists in Valparaiso.

8. Baker, J. A. 1944, J. Exper. Med. **79**: 159–172.
9. Nigg, C. and Eaton, M. D. 1944, J. Exper. Med. **79**: 497–510.
10. Hanks, J. H. and Wallace, R. E. 1949, Proc. Soc. Exper. Biol. & Med. **71**: 196–200.

ute for 1½ inches with a slight rotating motion.

Staining procedures.—The cultures were fixed in absolute methanol for at least 10 minutes, placed in May-Gruenwald stain for 10 minutes and Giemsa, diluted 1:10 with distilled water buffered to a pH of 6.8, for another 20 minutes. The cultures were rapidly destained in acetone, dehydrated and cleared in several changes of acetone-xylene mixtures and xylene and mounted on slides with permount.

EXPERIMENTAL PROCEDURES AND RESULTS

Cultivation of chick entodermal cells

Cultures of entodermal cells were obtained from blastoderms and yolk sacs of chick embryos varying in age from 15 hours to 18 days. The important dissimilarities which were encountered among the various cultures could be attributed to differences in the age of the embryos, the embryological location of the explanted tissues, and, to a lesser degree, the cultural procedures used. A study of the various cultures described below led to the selection of a type which seemed to be the best suited for quantitative virus studies.

1. Vascular yolk sacs of embryos 3 to 18 days old.—The entodermal cells of the intermediate age group of embryos, 8 to 12 days old, exhibited only moderate activity in tissue culture. During the first 2 days the cells migrated towards the periphery of the explants and the rounded edges of their cytoplasms protruded. Active migration into the surrounding areas was rarely noted before 48 hours and usually occurred between the 3rd and 4th day. The zones of outgrowth then increased during the following 4 to 6 days and the colonies reached maximum diameters of 5 to 6 mm. Since mitotic figures were rarely seen, outgrowth was probably due almost entirely to migration. Expansion of the zones of ourgrowth continued only as long as living cells were present in the explants. The cells remained in good physiological condition for about 15 days, then began to degenerate, and in about a month finally disintegrated. Destruction of entodermal cells was sometimes followed by growth of connective tissue cells.

The entodermal cells retained most of their original characteristics in vitro. They were very large, averaging 120 by 80μ in size and triangular or polygonal in shape (fig. 1). The large nucleus contained fine chromatin granules and one or two spherical or rod-shaped nucleoli. The cytoplasm was almost entirely vacuolated, with somewhat larger vacuoles near the center. Strips of unvacuolated cytoplasm could be detected either surrounding the nucleus or at the periphery of the cells. The cellular limits were not always well defined. Near the center of the colony the cells remained in close contact with each other, but towards the periphery they became more widely separated.

As shown in table 1, cells derived from the younger embryos were more active than those of the older groups. The chief differences consisted in the speed and number of cells migrating out of the explants. While the entodermal cells of the older embryos were morphologically similar, the cultures of the 3-day-old embryos harbored at least three epithelial cell types: vacuolated cells containing hyaline yellow yolk globules, as those previously described; cells similar in size and vacuolation but containing, in addition, at least one large refractile, granulated globule of white yolk; smaller cells with fewer vacuoles, probably of ectodermal origin. As already noted by Grodzinski,[11] after several days cultivation differences among the cell types became less marked. Cultures of young embryos also manifested a tendency to form sac-like structures.

TABLE 1.—*Colony size of explanted vascular yolk sacs of embryos of different age groups.*

Age of embryos (days)	Number of explants	Number of cultures: Porter flasks	Number of cultures: Roller tubes	Maximum diameter of colonies (mm): 8-6	6-4	4-2	2
3	1	35		4	24	6	1
8–12	6	39	58	2	32	62	1
15	1	19			2	17	
18	1	32				8	24

2. Blastoderms of embryos 15 to 24 hours old.—Large colonies were rapidly formed by explanted blastoderms. Migrating cells were detected as early as 18 hours after cultivation, and the majority of colonies achieved a diameter exceeding 8 mm by the 4th day. However, differences among cell types were even wider than in the cultures of 3-day-old embryos. Small, unvacuolated epithelial cells, probably of ectodermal origin, were usually found in the center of the

11. Grodzinski, A. 1930, Carnegie Institution of Washington, Pub. no. 132, Contrib. Embryol. 22: 155–172.

colony, while the vacuolated entodermal cells extended into the peripheral zone.

3. Avascular membranes of 3- to 5-day-old embryos.—The area of the yolk sac peripheral to the sinus terminalis of embryos 3 to 5 day old contained at least three zones which could be identified by their cultural characteristics.

A band, 2 mm wide, immediately surrounding the blood vessel, gave rise to colonies comparable in size to those of the vascular area. An intermediate band, 2 to 10 mm from the sinus terminalis, somewhat thicker and yellow in appearance, when explanted, produced a wide continuous sheet of vacuolated entodermal cells. Margins of the 11×22 mm coverslip were usually reached by the 4th or 5th day of cultivation. The yellow band continued distally into a thin white membrane containing smaller cells with fewer vacuoles. Explants from this area formed colonies of moderate size by projecting many narrow tongues of cells.

Of all the tissues studied, the intermediate area of the avascular membrane appeared, by far, to be the best suited for quantitative virus investigations, and it was therefore studied most thoroughly. The area was better recognized in 4- than in the 3-day-old embryos. Careful dissecting and plasma embedding of the explants were all important in successful colony formation. The size of the colony depended largely on the size of the explant. The shape of the colony was largely determined by the position in which the explant was embedded. A cleanly cut explant which remained in a flat position gave rise to just one layer of cells extending at the same speed in all directions. A jagged edge resulted in limited growth in that direction. A folded explant resulted in as many layers as folds. The importance of these observations lies in the fact that under suitable conditions it became possible to produce a single layer of just one morphological type of cell.

The cells were all large, vacuolated entodermal cells, often containing a large globule of white yolk. Unstained cells are shown in figure 2. In spite of their uniformity in size and general appearance, important differences in their physiological state were evident. Many cells contained large amorphous masses which stained deeply with the basic dyes. Others appeared to have a light matrix stippled with a more basophilic material. Still others contained small eosinophilic granules (fig. 3 and 4). The inclusions were most common in 5-day-old embryos. Cultures derived from 4-day-old embryos usually, but not always, were relatively free from cytoplasmic material (fig. 5). Inclusions were most numerous in the center of the culture and their size and number were considerably reduced by a few days cultivation. The nature of the cytoplasmic inclusions is not understood, but can be reasonably attributed to the glandular nature of the cells and the avascularity of the area.

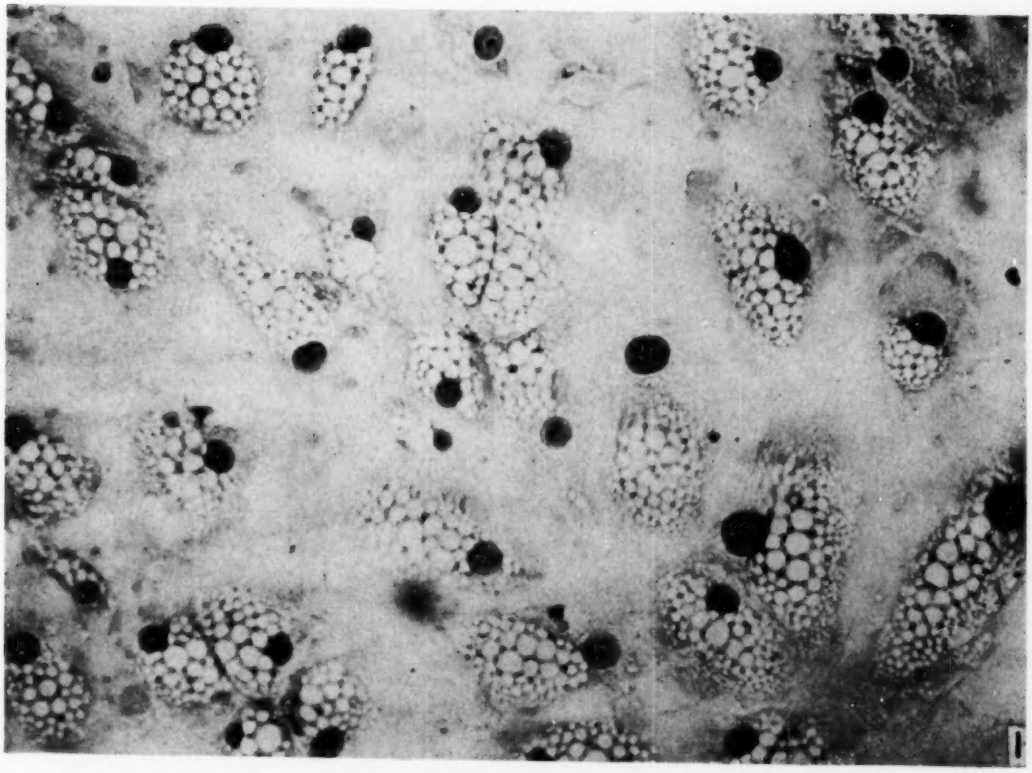
Cinematographic observations* indi-

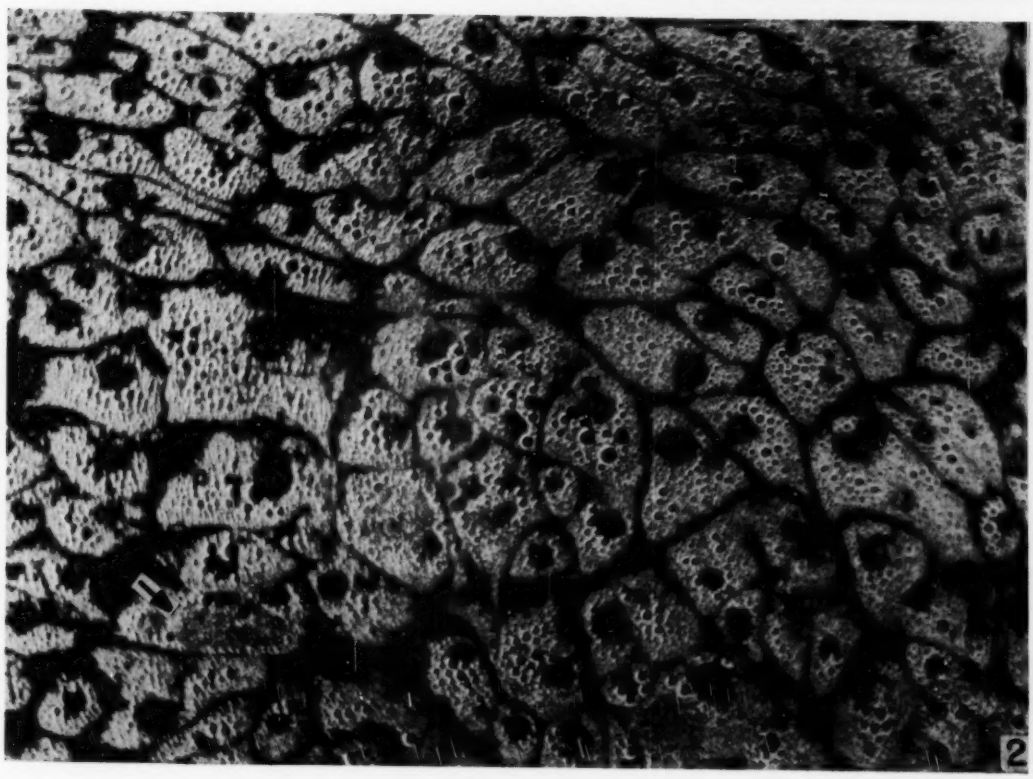
* We are indebted to Dr. Raymond G. Murray of the Department of Anatomy of Indiana University for the use of his cinematographic equipment.

FIG. 1–12.—Photomicrographs of chick embryo cells of normal (fig. 1 to 5) and infected (fig. 6 to 12) cultures prepared on coverslips in Porter flasks. Unless otherwise indicated, the cultures were fixed in absolute methanol and stained with May-Gruenwald-Giemsa stains. The photomicrographs were taken by Mr. Clarence M. Flaten of the Photographic, Audio-Visual Center of Indiana University.

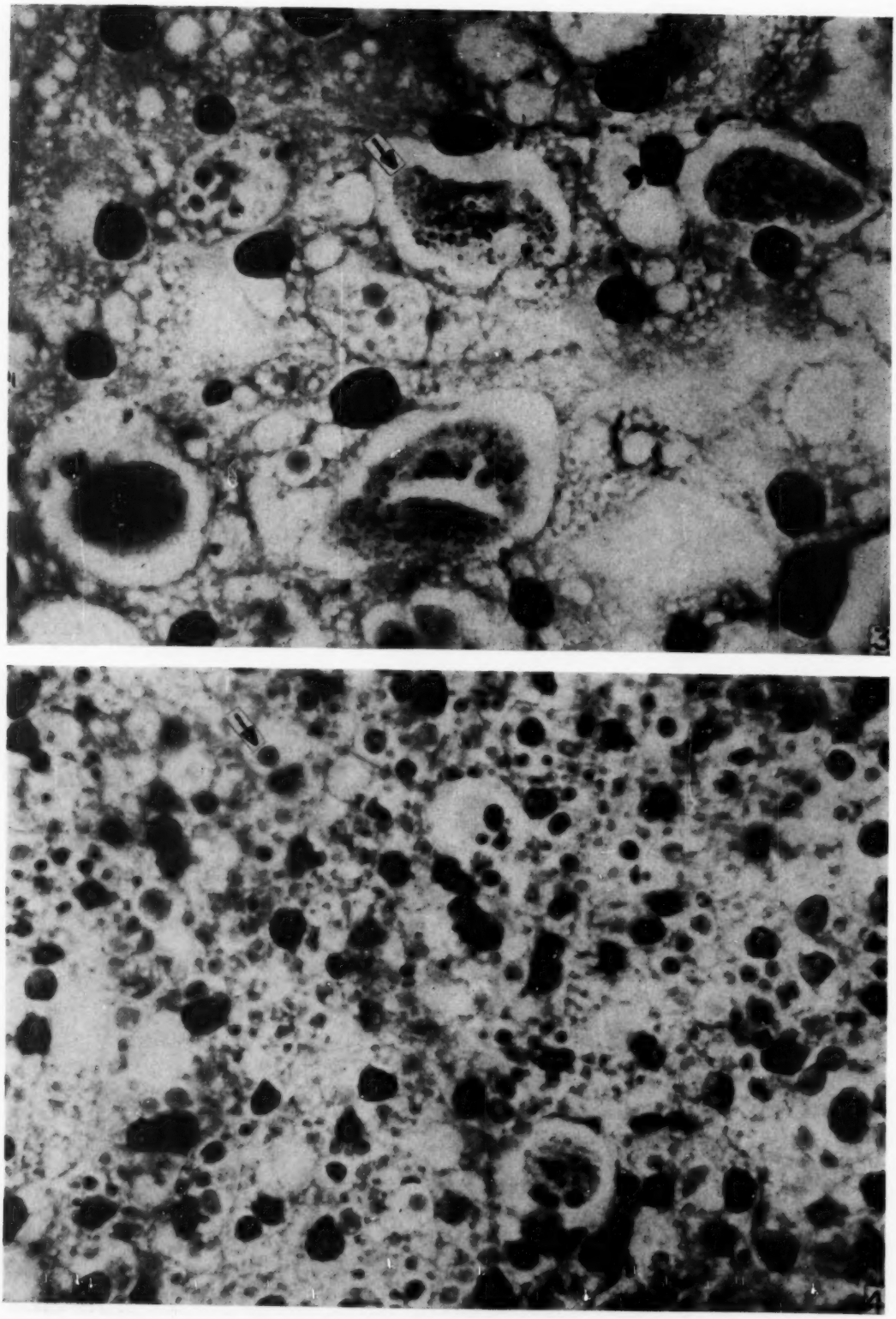
FIG. 1.—Eight-day-old yolk sac culture of seven-day-old embryo. The cells are well separated, as in most older cultures. Note yellow yolk vacuoles in the cytoplasm. Giemsa stain. ×225.

FIG. 2.—Living entodermal cells from a 4-day-old culture of the avascular membrane of a 4-day-old embryo. The culture was grown in a Porter flask on a coverslip which was transferred to a ring plate chamber a few hours before the photograph was taken. Note small yellow yolk granules and large finely granular areas of white yolk (such as indicated by arrow) in the cytoplasm of some of the cells. Darkfield. ×125.





FIGURES 1–2



FIGURES 3–4

cated that the growth of the colony was due primarily to centrifugal migration and expansion of tightly grouped cells. In stained cultures a small number of mitotic figures and a fair number of multinucleated cells were detected. The observations of Thomas[12–14] that the cells multiplied by an amitotic process and eventually gave rise to macrophages were not confirmed or disproved.

The entodermal cells were less affected by differences in cultural procedures than common chicken fibroblasts. A mixture of one part chicken serum and three parts Hanks' balanced salt solution appeared to be a satisfactory nutrient medium. The serological specificity of chicken serum did not appear to be essential, since the cells appeared to develop equally well in rabbit or sheep serum mediums. Colony development was somewhat less extensive when bovine serum ultrafiltrate was substituted for serum and very poor in plain balanced salt solution. The addition of chick embryo extract or bovine plasma albumin to final concentrations of 5 to 10% did not enhance or retard growth. Frequent changes of the nutrient fluid injured the cells, but because of the slow drop of the pH of the medium, they were not necessary.

A few unsuccessful attempts were made to eliminate the plasma clot and thus expose the whole area of the entodermal sheet to the supernatant fluid. The explants readily adhered to glass and formed colonies, but it was not possible to maintain the great majority of explants on the coverslips and colonies were often formed on the rounded edges of the Porter flasks. Colonies were readily formed under a sheet of perforated cellophane, but the cells proved to be very fragile and were severely damaged by subsequent handling. Embedding the explant in a small plasma clot, barely sufficient to insure adherence of the explant to the center of the coverslip, proved to be the most satisfactory method. The plasma was usually readily lysed by the migrating entodermal cells and did not effectively prevent viral penetration of the cells.

Exposing the cells alternately to fluid and gaseous phases, as in roller tubes and rotating Porter flasks, stimulated colony formation. In Porter flasks migration of the cells was retarded by the edges of 11×22 coverslips, but often a second layer was formed on the opposite side of the coverslips. In Maximov slides the activities of the cells were considerably reduced in most cases. Good results were obtained, however, when single explants were enclosed with 0.5 ml of nutrient fluid in chambers formed by two 40×40 mm coverslips and a 35 mm stainless steel ring-plate. The colony developed on the lower coverslip to a size almost comparable to that of the Porter flask cultures.

Growth of the agents of feline and murine pneumonitis in tissue culture

The agents of feline and murine pneumonitis could readily be grown in entodermal cell cultures. Entodermal

12. Thomas, J. A. 1934, Compt. rend. Soc. de biol. **117**: 758–760.
13. Thomas, J. A. 1935, Compt. rend. Acad. de sc. **201**: 988–990.
14. Thomas, J. A. 1936, Arch. f. exper. Zellforschung, **19**: 299–323.

FIG. 3 and 4.—Cells from avascular membranes from 3- and 5-day-old chick embryos, respectively, explanted 2 days previously. Note very large granular cytoplasmic inclusions in figure 3 and small inclusions littering the field in figure 4 (examples given by arrows). The inclusions can readily be distinguished from virus vesicles (fig. 6 to 12) because they have slightly different affinities for dyes and do not contain homogeneous particles of the size of elementary bodies. ×435.

and some of the ectodermal cells appeared to be the only ones capable of supporting the growth of these viruses in tissue culture. Various attempts to grow them in other types of cultures, such as embryonic chick heart or murine lung, failed.

The cultures could be infected with either virus by several methods. Bathing the explants in infected fluid prior to explantation usually required at least 1000 chick embryo infectious units. The same amount was needed when virus was added to the nutrient fluid immediately after preparation of the culture. More effective inoculation was accomplished when the virus was added to a well-developed colony. Under appropriate conditions, as shown in a later section, one chick embryo infectious unit was sufficient to initiate infection of these cultures.

Demonstration of the growth of these viruses was accomplished by: (a) recovery of the virus from the supernatant and further passagé in tissue cultures or chick embryos, (b) lysis of the host cells and disintegration of the colonies, and (c) detection of typical inclusion bodies in stained cultures.

As shown in table 2, feline pneumonitis virus injected into cultures rapidly disappeared from the supernatant and could not be detected 24 hours after inoculation. It reappeared, however, at 48 hours and its titer slowly increased until the colony disintegrated. The relatively small number of host cells and the instability of released virus in the culture medium at 37 C were undoubtedly important factors in limiting the titers which were obtained in these cultures. Attempts have not yet been made to increase the possible yield of virus. However, the various phases of feline pneumonitis virus infection are now being investigated in detail and will be reported in a later publication.

TABLE 2.—*Infectivity of the supernatant fluids withdrawn at various intervals of time after inoculation of the cultures with feline pneumonitis virus.*

Time interval (hours)	Number of specimens tested*	Embryos dying	Average day of embryo death†
		Embryos inoculated	
0	2	11/11	5.1
24	4	3/20	10.4
48	4	18/20	7.5
72	3	15/17	6.6
96	4	23/23	5.9

* Each specimen was diluted 1:10 and inoculated into 5 or 6 chick embryos.
† Determined by twice daily observations for 10 days and by arbitrarily giving 10.5 as the day of death to surviving embryos.

Infection of the colony was followed 4 to 6 days later, depending on the concentration of virus, by lysis. Microscopic examination showed lysis of isolated cells after 2 days. The lysing cells released enough virus particles to infect a larger number of cells, and eventually, by 4 or 6 days, the whole colony was involved and disintegration was followed macroscopically. Observation of infected unstained and stained cultures revealed few degenerating cells. It appeared that destruction of the host cells occurred suddenly. Lysis was possibly due to a disturbance of the equilibrium of the surface tension of the fat globules and that of the virus laden protoplasm.

The inclusion bodies formed by feline and murine pneumonitis viruses were entirely analogous to those described in chick embryos.[3,4] They appeared first as small clusters 12 to 18 hours after inoculation and then readily developed into large vesicles. The full size of the vesicles was reached at 36 to 48 hours, just prior to cell lysis. At later time intervals all developmental stages were detected (fig. 6 to 11).

Differences between feline and murine pneumonitis virus structures were obvious. In entodermal cells, feline pneumonitis virus (fig. 6 and 7) developed in the protoplasmic strands without greatly distorting the host cells. The surface

tension of the host protoplasm was greater than that of the virus vesicle. Murine pneumonitis clusters and vesicles, on the other hand, because of a higher surface tension, preserved an oval or rounded structure (fig. 9 and 10) even in the vacuolated protoplasm of their host cells. The vesicles often pressed against nuclei and distorted them (fig. 10). In ectodermal cells (fig. 8 and 11) the two viruses formed smaller vesicles and, probably because of the absence of vacuoles, differences due to forces of surface tension were not marked.

The various types of cells obtained from the yolk sac and blastoderm of chick embryos appeared to exhibit different degrees of susceptibility to infection. The highly vacuolated entodermal cells were, by far, the most susceptible. They were the first to be infected in blastoderm cultures which contained both vacuolated and unvacuolated cells. The infection of the unvacuolated cells usually followed the massive release of virus from the vacuolated cells. A similar difference in susceptibility was noted between the cells of the intermediate and distal areas of the avascular membranes of the 4-day-old chick embryos. Cells containing cytoplasmic inclusions were more resistant to infection than those with a relatively free cytoplasm.

The infected cell count

The foregoing observations formed the basis for the development of a new method of viral titration, consisting of enumerating the cells initially infected. The agent of feline pneumonitis develops for 48 hours in the same cell before lysing it and infecting other cells. Therefore, assuming that one successful viral particle is sufficient to infect a cell, the number of infected cells during this 48 hour cycle will be proportional to the concentration of the virus inoculated, provided the percentage of infected cells is small.

In short, this method consists of titrating an unknown specimen of feline pneumonitis virus by inoculating entodermal cell cultures with a single dilution of the specimen. About 30 hours after inoculation the cultures are fixed, stained and examined for number of infected cells. The concentration of virus is determined by the count, multiplied by the original dilution, and can be expressed in terms of infectious units. Assuming that viral infection follows the law of Poisson's distribution, the number of ID_{50} (50% infectivity) units can be calculated by applying the formula:

$$1\ ID_{50}\ \text{unit} = 0.7\ \text{infectious unit}$$

In the development of the infected count method, the following observations were of practical importance:

1. Single layers of the same type of entodermal cells, as free as possible of cytoplasmic inclusions, were, of course, essential for accurate counting. Occasionally, the presence of a second layer of entoderm over part of the colony did not interfere with visibility. The explant itself was not an important source of error, as it absorbed few viral particles.

2. The colonies continued to develop in size for about a week, but their areas were sufficiently large for viral inoculation by the 4th day. Signs of degeneration were encountered among cells preincubated for longer periods of time and affected the accuracy of the infected cell counts. The cultures were inoculated with pipettes or syringes by adding 0.25 ml of the appropriate dilution of virus to each culture. The final volume of fluid in the culture was either 1.00 to 1.25 ml. Care was taken to have the same final volume of fluid in each culture of the same experiment. To avoid as many variables as possible in the composition of the nutrient fluid, viral specimens were usually diluted in the pooled supernatant fluids of discarded cultures.

3. Although the average developmental cycle of feline pneumonitis virus required 48 hours, some of the cells were lysed much sooner. Therefore, the most appropriate time for fixing and straining the cultures were approximately 30 hours after inoculation.

4. Some cells contained a single large vesicle and others appeared to have two or more small

TABLE 3.—*Variations in infected cell counts among identically treated cultures.*

Number of cultures in each group	Number of groups	Mean standard error		
		Low	High	Average
3	6	2.6	6.6	4.6
4	20	3.3	17.0	6.6
5	3	3.3	7.4	5.0
6	3	3.8	5.6	4.4
8	2	3.8	5.5	4.7

TABLE 4.—*Relationship between infected cell count and viral concentration.*

Exp. no.	Group	Viral concentration	Mean ± S.E.	Mean ± S.E. Concentration factor
61	1	C* ×1	34 ± 2	34 ± 2
	2	C ×2	80 ± 15	40 ± 8
	3	C ×4	134 ± 20	34 ± 5
	4	C ×8	284 ± 54	36 ± 7
74	1	C† ×1	84 ± 18	84 ± 18
	2	C ×3	250 ± 24	83 ± 8

* Virus pool diluted $10^{-4.3}$.
† Virus pool diluted $10^{-3.9}$.

ones (fig. 12). It was not clear whether or not these multiple structures had derived from the same infectious unit. However, in those cultures where the infected cells were only a small fraction of the total number, it appeared justified to assume that this was true. Some of the multiple vesicles were possibly due to intracellular breaking up, or just strangulation, of a large vesicle. Microscopic observations in some cases suggested this possibility (fig. 12). Therefore, the count was based on the number of infected cells rather than on the number of virus vesicles.

5. The infected cells were readily recognized at high dry power and even low magnification. When the cells were relatively free of cytoplasmic inclusions and well stained, observation with a 4 mm objective was sufficient for counting (an 8 mm objective was used for the photograph in fig. 12). But for clear differentiation of the vesicles from other basophilic structures, resolution of the individual granules was necessary. This could be accomplished only with an oil immersion objective, but magnification was reduced by the use of a low power ocular (5 or 6×). The number of infected cells which could be counted accurately varied with the size of the colony. Usually, counts of as many as 500 to 1000 infected cells were reliable. When the cultures contained more than 1000 infected cells, the chances of counting the same structures twice became greater.

6. Variations in counts among identically treated cultures were fairly large, as indicated in table 3. The error has been reduced in recent experiments. In spite of these variations, usually 3-fold and often 2-fold differences in counts carried out in quadruplicate were significant.

The assumption that infection of a cell was caused by a single virus particle appeared valid, provided the counts were not very high. As indicated in table 4, with individual counts not exceeding 400, a linear relationship was obtained between concentration of virus and infected cell count.

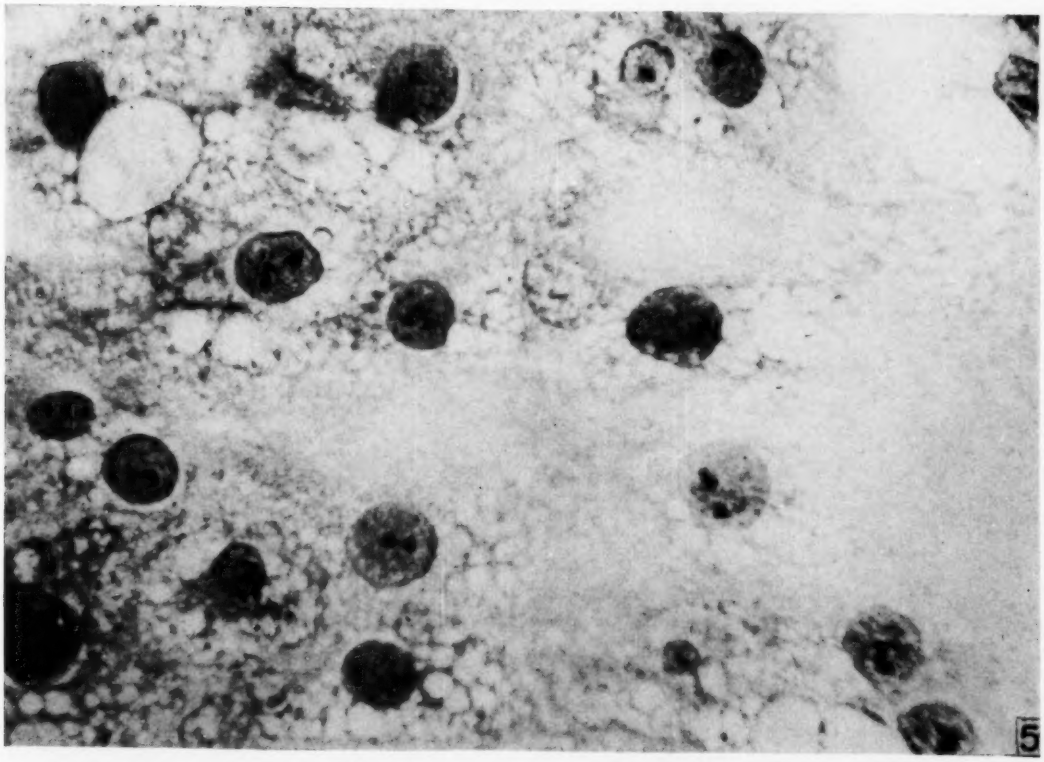
The correlation between infected cell count, tissue culture infectivity titration, and routine chick embryo titration is shown in table 5. The sensitivity of tissue culture titration methods varied. In one experiment (exp. 43) the infected cell count appeared to be less sensitive and in the other (exp. 60) about as sensitive as the routine chick embryo titration method. This difference could be attributed to slight variations in the procedure. There also was good correlation between the counts and the tissue culture infectivity titrations. The apparent discrepancy noted in experiment 60 is not significant. The results indicated that, under appropriate conditions, the infected cell count was just as sensitive and considerably more accurate than the chick embryo technique.

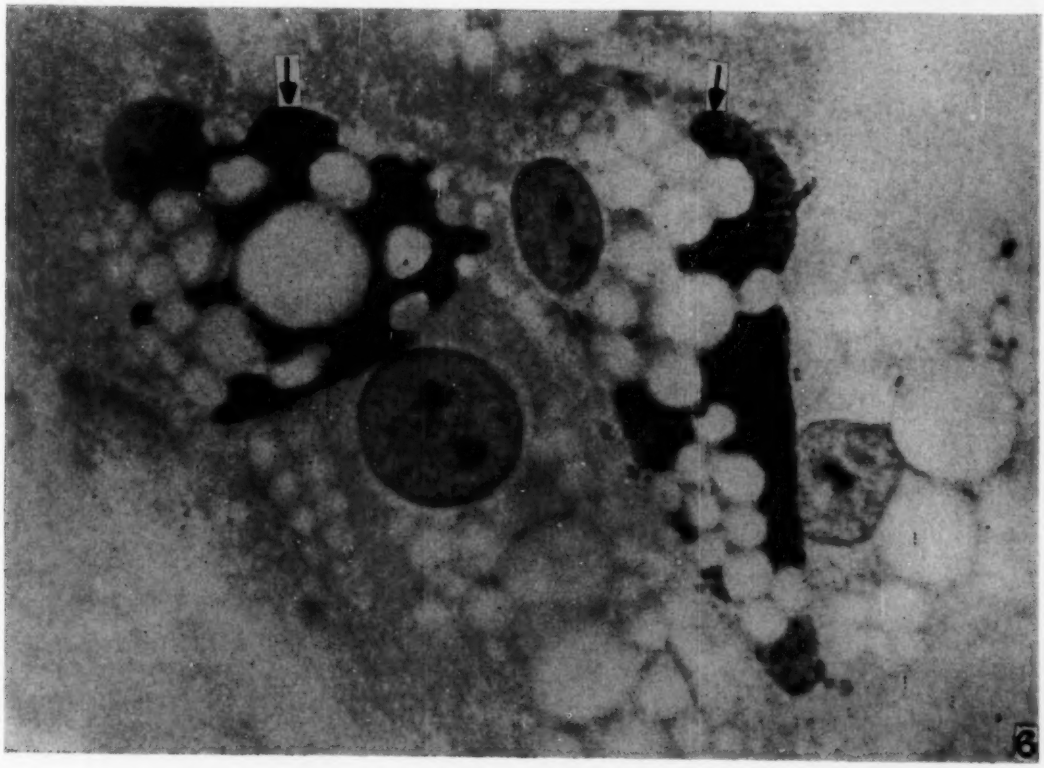
Factors affecting the infected cell count

Differences in susceptibility to feline pneumonitis infection among various

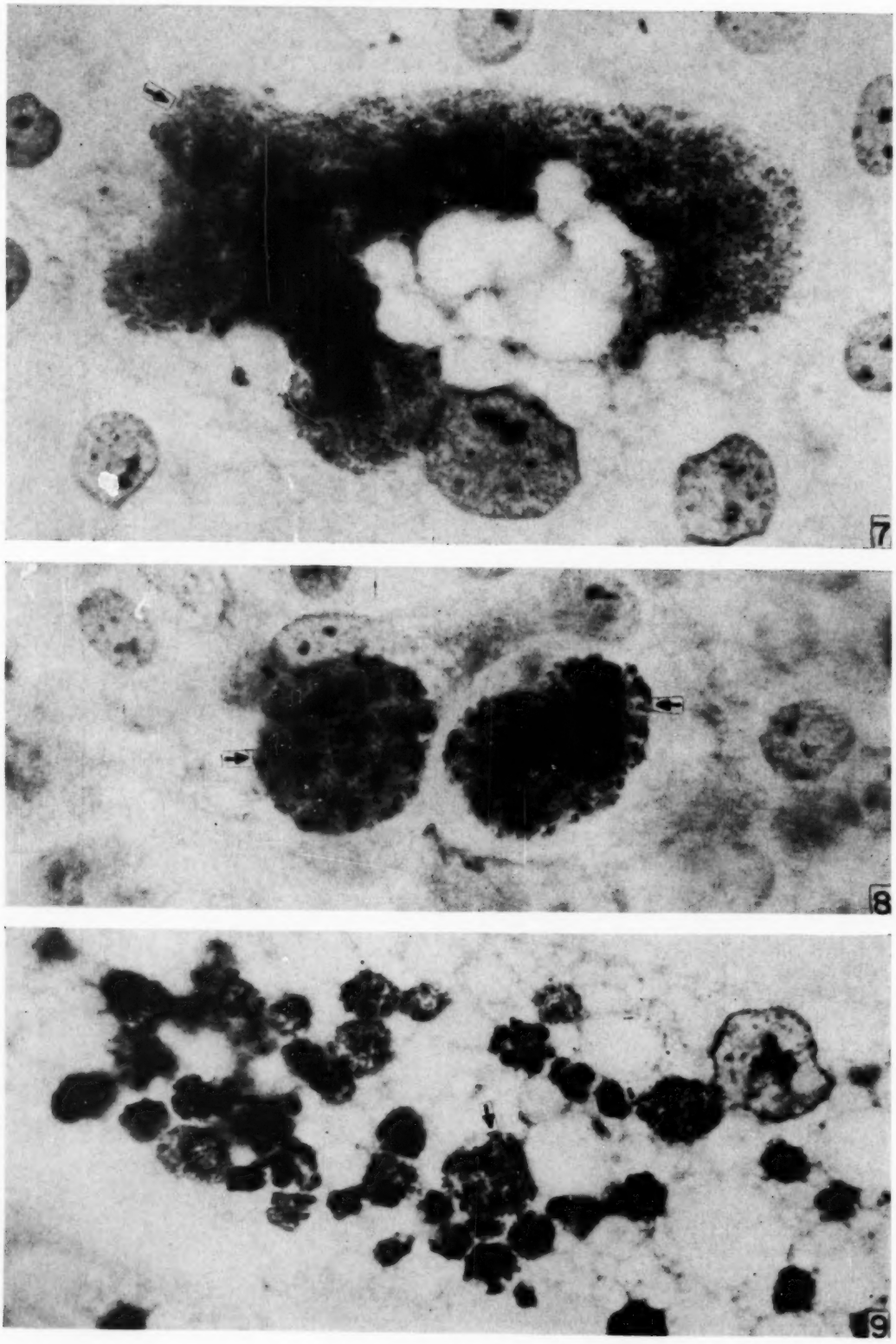
FIG. 5.—Cells free from inclusion bodies from the avascular membrane of a 4-day-old chick embryo, explanted 2 days previously. ×435.

FIG. 6.—Feline pneumonitis virus in blastoderm culture of 1-day-old embryo. Note two infected cells. The virus (arrows) appears to have developed along the protoplasmic strands surrounding the vacuoles. ×1040.





FIGURES 5–6



FIGURES 7–9

TABLE 5.—*Virus titrations by different methods.*

Exp. no.	Virus dilution	Infected cell count	Tissue culture infectivity titration (ID_{50})†	Chick embryo titration (LD_{50})†
43	4.0	18±4	++++	+++++
	5.0		++++	+++++
	6.0		+---	++++-
	7.0		----	-----
	8.0		----	-----
	ID_{50} or LD_{50}*	5.4	5.8	6.5
60	4.0	407±9	++++	
	5.0		++++	
	6.0		++++	+++++--
	7.0		+++-	++-----
	8.0		++--	-------
	ID_{50} or LD_{50}*	6.76	7.8	6.5

* Expressed as the inverse of the logarithm of the dilution.
† Infection or death indicated by +, freedom from infection or survival by −.

types of epithelial cells or among cells with or without cytoplasmic granules have already been noted. Most of these differences were quite obvious and did not require the counting of the infected cells.

Individual embryos exhibited varying degrees of susceptibility. Three groups of cultures, each derived from a different embryo, were inoculated with the same amount of virus. As indicated in table 6, differences in infected cell counts among the three groups were significant. The results suggested a physiological or genetic difference among the embryos, although differences in the handling of the explants from each embryo during the cultural procedure could not be excluded.

Hanks[15] clearly pointed out that serum, although the best nutrient for cultured cells, often contained viral inhibitors and usually did not enhance the growth of viruses in tissue cultures. With this in mind, several attempts were made to increase the infected cell counts by finding an adequate substitute for serum. Efforts were also made to further enrich the medium and to increase the stability of the virus prior to its absorption into the cells.

The results of some of these experiments are shown in table 7. The infectivity of feline pneumonitis virus was not increased by the substitution of various reagents for serum. Some of these, such as 10% bovine plasma albumin or 35% bovine serum ultrafiltrate, proved to be just as satisfactory. Others, such as a mixture of chicken serum and Fischer V614 synthetic medium, proved to be slightly inhibitory. The inclusion of chick embryo extract in the medium proved to be with-

TABLE 6.—*Infected cell counts of cultures from individual embryos.*

Exp. no.	Embyro	Number in group	Mean±S.E.
53	1	4	40± 9
	2	4	89±16
	3	4	160±21

15. Hanks, J. H. 1952, Bact. Proc. 99.

FIG. 7.—Feline pneumonitis virus vesicle (arrow) almost completely filling the cytoplasm of an entodermal cell of a blastoderm culture of a 1-day-old embryo. ×1040.

FIG. 8.—Ectodermal cells of the same type of culture as in figure 7, containing two rounded vesicles (arrows) of feline pneumonitis virus. ×1040.

FIG. 9.—Entodermal cell containing many small vesicles of murine pneumonitis virus. Note that, in contrast to feline pneumonitis virus, they all maintain a rounded or oval appearance. A typical vesicle is indicated by arrow. Entodermal cell culture of 1-day-old embryo. ×1040.

TABLE 7.—*Infected cell count of cultures containing different nutrient fluids*

Exp. no.	Group	Number in group	Nutrient fluid*	Mean ±S.E.
47	1	5	$CS_{10}EE_{10}$	441±60
	2	4	$CS_{10}V614_{70}$	171±38
50	1	3	$CS_{10}EE_{10}$	240±47
	2	3	CS_{10}	258±62
	3	3	$CS_{10}EE_{10}PA_{10}$	158±24
	4	3	PA_{10}	190±64
51	1	4	CS_{10}	34± 8
	2	4	PA_{10}	43±11
	3	3	UF_{80}	47±18
	4	3	BSS	1± 1

* The abbreviations are explained in *Materials and Methods*. The number following each symbol indicates the percentage of the reagent in the nutrient medium. All reagents were diluted in BSS.

out effect. The same was true of bovine plasma albumin.

In general, procedures which were detrimental to the cells also prevented infection. As shown in table 7, suspending the cells in balanced salt solution, for example, greatly reduced the count. Renewing the nutrient fluid at the time of inoculation had the same effect. This is shown by a comparison of experiment 50 and 51. The cultures in the two experiments were inoculated with the same amount of virus, but the nutrient fluids in experiment 51 were changed during the inoculation, while in experiment 50 they were changed the day before inoculation. The nature of the fluid used as diluent for the virus also influenced the count, as shown in table 8. Balanced salt solution with or without 5 or 10% bovine plasma albumin appeared to be a suitable diluent. However, a high concentration of bovine plasma albumin (20%), allantoic fluid, and possibly nutrient broth were slightly inhibitory.

Thermal inactivation of the virus was not directly studied, but undoubtedly played an important role in many of the experiments. The infected cell counts were usually higher when lengthy manipulations were not required or when the virus was kept in an ice bath during the entire period of dilution and inoculation.

Several attempts were also made to increase the rate of contact between virus and host cells (table 9). Incubating the inoculum with trypsin, 1 mg per ml, for 1 hour at 25 C did not increase the count. However, the counts were significantly increased by gently shaking the cultures. They were higher than those left undisturbed and even those which were placed in a tissue roller.

The studies described above led to the increase of the sensitivity and accuracy of the test, but many important factors remained to be investigated.

TABLE 8.—*Infected cell counts of cultures inoculated with virus diluted with different reagents.*

Exp. no.	Group	Number in group	Virus diluent*	Mean ±S.E.
47	1	5	BSS	537±133
	2	5	PA_5	441± 60
	3	5	NB	250± 75
48	1	3	BSS	285± 46
	2	4	PA_5	168± 22
	3	4	PA_{10}	299± 79
	4	4	ALL	109± 12
49	1	4	PA_{10}	82± 18
	2	4	PA_{10}	2.5± 1

* The abbreviations are the same as for Table 7. NB =nutrient broth. ALL =allantoic fluid.

DISCUSSION

The main effort of this work was directed towards finding a quantitative method for the study of the kinetics of viral infection. Many problems concerning the host cell as well as the virus

TABLE 9.—*Infected cell counts of cultures treated by different methods.*

Exp. no.	Group	Number in group	Procedure	Mean ±S.E.
49	1	4	Incubation of virus pool with trypsin*	113± 34
	2	4	Incubation of virus pool with BSS*	82± 18
52	1	4	Same as 49, group 1	879±219
		4	Same as 49, group 2	783± 86
56	1	6	Cultures not moved	100± 18
	2	6	Cultures rolled	147± 39
	3	6	Cultures shaken	310± 73
63	1	8	Same as 56, group 2	262± 69
	2	8	Same as 56, group 3	455± 99

* For 1 hour at 25 C.

were merely touched upon and deserve further attention.

The entodermal cell is undoubtedly one of the most important cells for the growth of rickettsiae and many viruses.[16] However, very little information is as yet available on its properties. Thomas,[12–14] after an extensive study, came to the conclusion that these cells eventually gave rise to macrophages. He also believed that mitosis was not its principal mode of multiplication. Instead, multiplication took place by a process called meroamitosis, which resulted in the production of many small nuclei from one large one. Nuclear division was not always accompanied by division of the cytoplasm. Moulder, McCormack and Itatani[17] indicated that normal yolk sacs utilized fatty acids rather than glucose as a source of energy. All this, as well as our observations, point to the fact that these cells differ from others in many important aspects.

The two viruses studied, feline and murine pneumonitis, developed in their host cells without producing any obvious injury until the time of lysis. The mechanism of lysis, and especially the host-parasite relationship prior to lysis, is not understood. Zahler and Moulder[18] have shown that feline virus infection resulted in important changes in the metabolic patterns of the cells. Finding the physiological counterpart of this biochemical phenomenon, in terms of loss or acquisition of specific functions by the cell, is another problem which needs to be investigated.

The single layer of entoderm cultivated in vitro appeared ideally suited for a study of the morphological characteristics of viruses of this group. The procedure for the preparation of the cultures is simpler than that involved for the preparation of tissue sections, and the cells and their inclusions are seen in their entirety.

Finally, the study of the numerous factors which influence viral infection must be pursued. In our limited experience, some were obviously important, and others cannot be directly held responsible for the results obtained. For example, the inhibitory effect of some of the reagents can conceivably be attributed to a change in viscosity of the medium, and, consequently, to a changed rate of host cell-virus contact. Not until all the major factors are studied will it be possible to discern those which are of primary significance.

The results obtained by the infected cell count method suggest that feline pneumonitis virus differs in many respects from bacteriophages and the agent of Western equine encephalomyelitis, recently used for quantitative studies by Dulbecco.[2] The most important differences are the relatively larger errors encountered in quantitative estimations of feline pneumonitis virus and the ease with which the infected cell counts are modified by changes in experimental procedures. Some of these variations in infected cell counts can readily be explained. The rate of thermal inactivation of extracellular feline pneumonitis virus is high,[6] and a delay in contact between virus and host cell reduces infectivity rapidly. Host specificity, as evidenced by our work, is far greater than that of Western equine encephalomyelitis. A high rate of contact between the virus and the host cell is necessary for efficient inoculation.

The most likely explanation for the observed characteristics of feline pneumonitis virus infection is that only a

16. Rivers, T. M. 1952, Viral and Rickettsial Infections of Man, ed. 2, Philadelphia, J. B. Lippincott Company.
17. Moulder, J. W., McCormack, B. R. S. and Itatani, M. K. 1953, J. Infect. Dis. **93**: 140–149.
18. Zahler, S. A. and Moulder, J. W. 1953, J. Infect. Dis. **93**: 159–165.

small percentage of the larger number of virus particles injected into a culture succeeds in infecting cells. By appropriate changes of the procedure more particles invade cells, but even under the best conditions, it is probable that infection is accomplished by relatively few particles. According to the studies of Zahler and Moulder,[18] about 100 particles are necessary to initiate infection of a chick embryo. About the same number is probably necessary to start infection of cultured entodermal cells by our techniques.

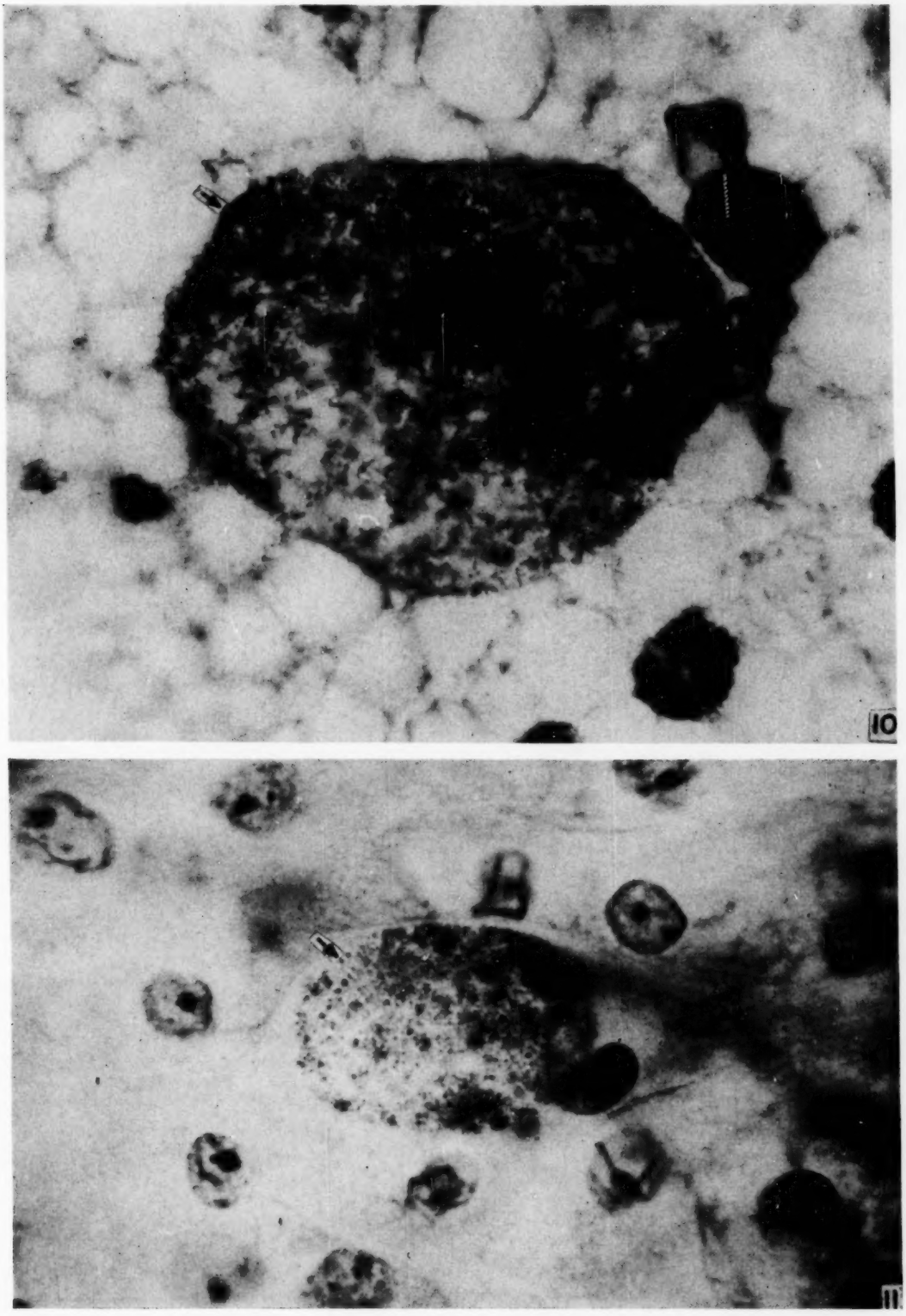
Feline pneumonitis virus resembles a great many animal viruses. Unlike the infection of the chick embryo by Western equine encephalomyelitis virus, which probably requires the introduction of just one infectious particle, most viruses require, depending on the host and route of inoculation, a variable number of particles to insure the success of one. A similar phenomenon is also encountered among plant viruses. When quantitative determinations are made by inoculation of leaves,[19] the use of abrasives increases the lesion counts by favoring the chance contact between virus and host cell. Although counting the cells infected with feline pneumonitis virus is somewhat less accurate than the count of plaques produced by bacteriophages or Western equine encephalomyelitis virus, it has the advantage of representing a more typical phenomenon of animal virus infection.

The infected cell count method offers several advantages over the chick embryo titration. By the latter method viral growth is determined by the use of at least 40 chick embryos for each specimen. Differences in concentrations among virus specimens must be at least 3-fold and often 10-fold to be significant. On the other hand, in the count method a single chick embryo will usually provide 24 good cultures. Thus, if 4 cultures are used for each specimen, 6 specimens can be titrated with a single chick embryo, and the accuracy is increased to the point where 2- to 3-fold differences become significant. The accuracy can be further increased by using more cultures for each specimen. Titrations carried out at different times and with different embryos can be compared if identical procedures have been used and differences among individual embryos are taken into account.

The amount of labor involved in the two procedures is about the same. About a full working day is required to prepare 48 cultures from 2 chick embryos. Inoculation, which is usually carried out 4 days later, with 1 dilution of 12 viral specimens requires only a few minutes. Fixing and staining 48 cultures, about 30 hours later, requires about 2 hours work. Counting infected cells requires about 10 minutes per culture with a 4 mm objective and a 6× ocular. Although precision is increased with an oil immersion lens, the counts with the high dry lens are lower in proportion and satisfactory in most cases. In all, the count procedures for 12 viral specimens require the equivalent of 2 or 3 days of a full time technician. This would replace the handling of 480 chick embryos by the routine titration procedure. An additional very important advantage of the infected cell method is that the results can be obtained within 48 hours after inoculation, while the chick embryo titration method usually requires 10 days.

19. Bawden, F. C. 1950, Plant Viruses and Virus Diseases, Waltham, Chronica Botanica Company, p. 63.

FIG. 10 and 11.—Murine pneumonitis virus in entodermal cell cultures of 1-day-old embryo. ×1040.
FIG. 10.—Large vesicle (arrow) in intodermal cell pressing against the nucleus.
FIG. 11.—Vesicle (arrow) in ectodermal cell.



FIGURES 10–11

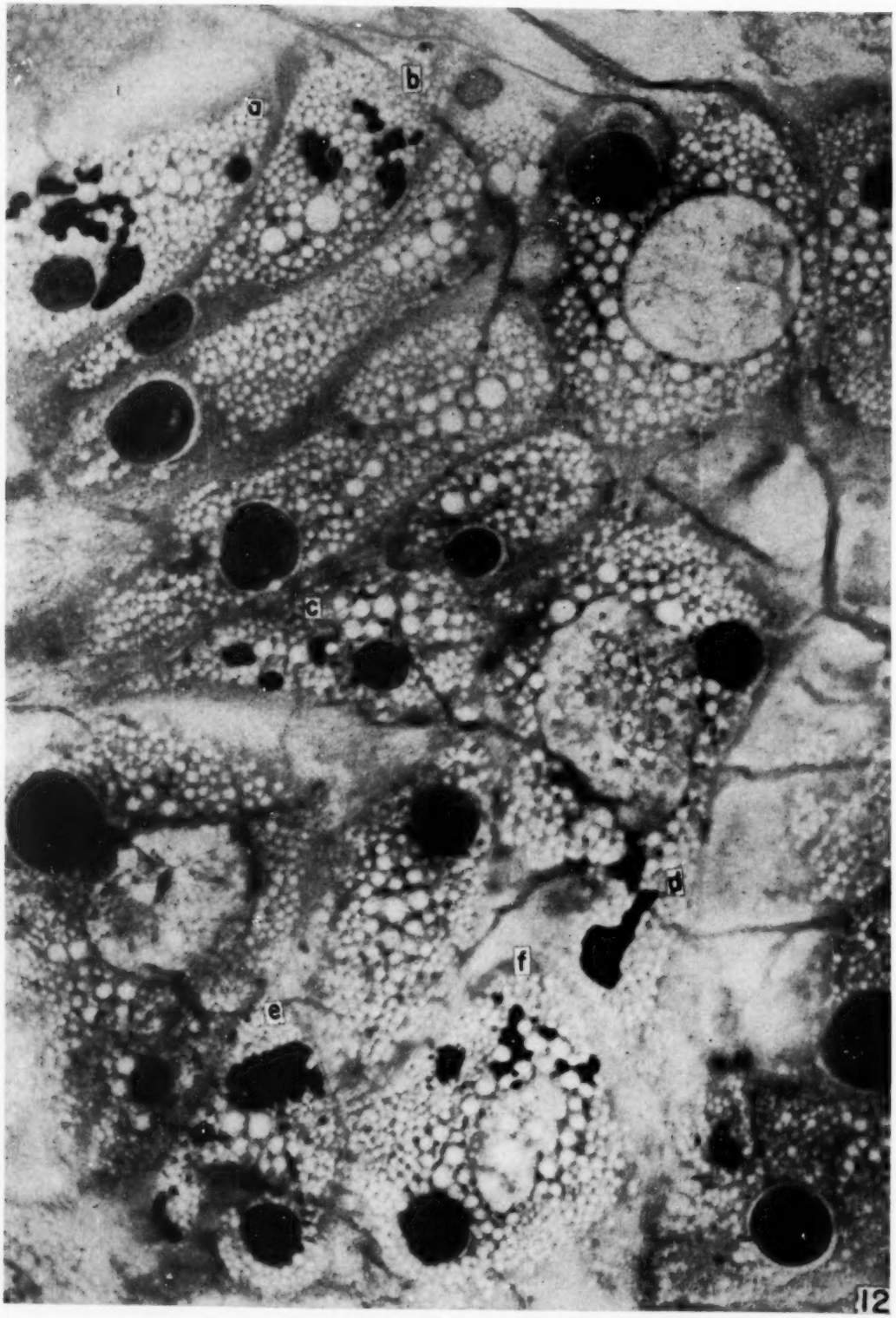


FIGURE 12

The most important advantage of entodermal cell cultures, of which the infected cell count is an application, consists of the fact that it greatly simplifies the study of the relationship of the virus to its host cell. In fact, the methodology of bacteriophage research is closely approximated. The time of exposure of the cells to infection, temperature of incubation, the composition of the nutrient medium and many other environmental factors can be carefully controlled, and their effect on infectivity rapidly determined. The mechanism of infection can thus be studied more directly.

SUMMARY

A tissue culture technique for virus assay based on the counting of infected cells has been developed. The agent of feline pneumonitis was used as the test organism, with the entodermal cell of the chick embryo as its host cell. Since feline pneumonitis had not previously been cultivated in vitro, this investigation also entailed the study of the characteristics of entodermal cells, the behavior of the virus in tissue culture, and a survey of the principal factors responsible for high infectivity.

The best suited cells for this study were found to be those derived from an intermediate area of the avascular zone of the yolk sac of 4-day-old chick embryos. Explants from this area, 2 mm in diameter, under appropriate conditions each gave rise to a single layer of large vacuolated cells covering an area of about 1 cm^2.

Feline pneumonitis virus readily developed in these cells and formed vesicles identical to those previously described in the yolk sac of chick embryos. Infected cells lysed 36 to 48 hours after inoculation and new cells were infected. The cycle was repeated until all the cells in the colony were lysed.

The infected cell count titration was based on the number of cells which were initially infected. Well developed cultures were inoculated with a single dilution of an unknown specimen of virus. Sometime during the first cycle of viral development, usually 30 hours after inoculation, the cultures were fixed and stained and the number of infected cells counted. The virus titer was then calculated from the count and the dilution used for inoculation.

The procedure has been perfected to become as sensitive as the chick embryo technique and considerably more accurate. Counts carried out in quadruplicate revealed threefold and often twofold differences in titer.

Application of this method indicated that individual embryos varied in susceptibility to infection. Serum, or an adequate substitute, was essential for high infectivity. Procedures which increased the rate of chance contact between virus and host cells, such as shaking the cultures, significantly increased the cell count. The entodermal cell cultures and the infected cell count method promise to be very useful for the study of many problems of host-viral relationship.

FIG. 12.—Entodermal cell culture infected with feline pneumonitis virus. The photograph was taken with a 20× objective with a numerical aperture of 0.66. Virus vesicles can readily be recognized, although the individual elementary bodies are not resolved. Six infected cells can be counted in the field (indicated by a, b, c, d, e, f). In some cases a continuity is suggested among the virus structures in the same cell (d and f, possibly b, c, and e). In other cases the structures are well separated as if derived from different infectious units (a). Note the large white yolk-vacuoles in some of the cells. ×497.

ARTIFICIAL IMMUNITY TO *FASCIOLA HEPATICA* IN RABBITS

I. SOME STUDIES WITH PROTEIN ANTIGENS OF *F. hepatica*

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Resistance of an animal to a helminth infection may take the form of either true acquired immunity or of premunity. In the former the immunity is essentially a postinfection state in which resistance to reinfection is not dependent on the persistence of the initial infection; in the latter the resistance to reinfection depends on the persistence of the initial infection and is actually a resistance to superinfection.

One may attempt to produce true acquired immunity by two methods: first, by infection of the host with the parasite and ensuring elimination of the latter before reinfection, and secondly, by the injection of extracts of the parasite. The first method is preferable if the specific immunity is thought to be dependent on the stimulus of the excretory or secretory products of the worms; valuable results have been obtained by this method (see, for example, Fairley, Mackie and Jasudasan, 1930; Ozawa, 1930; Africa, 1931; Schwartz, Alicata and Lucker, 1931; Kagan and Lee, 1953), and Taliaferro (1940) has concluded that precipitins of functional importance in immune reactions of the host are formed in response to materials discharged by the worm. The second method is necessary if one wishes to study the immunological properties of specific fractions of the parasite, and to quote Craig and Faust (1951), "As yet very little critical work has been done in animal parasitology to determine the relative specificities of the protein, polysaccharide and cholesterol fractions of whole antigen. This field deserves much more thorough and careful investigation." Work with whole worm extracts has demonstrated the presence of acquired immunity in fascioliasis of rabbits (Kerr and Petkovitch, 1935), schistosomiasis of dogs (Ozawa, 1930), schistosomiasis of mice (Watts, 1949), *Cysticercus fasciolaris* infections of rats (Miller, 1932), and in haemonchosis of sheep (Stoll, 1942). Stewart (1950) failed to protect sheep against *H. contortus* or *Trichostrongylus* sp. by means of vaccines prepared from ground infective larvae.

The work described in this paper is part of an investigation to isolate and to identify as far as possible immunologically active fractions of the liver fluke *Fasciola hepatica* and, using these materials as immunizing antigens, to investigate their effectiveness in stimulating a resistance to the natural infection. The serums of rabbits infected with *F. hepatica* were found to contain precipitins which reacted with a protein extract prepared from the adult parasite, and it was therefore considered desirable to start the investigation with a study of the proteins of the parasite.

METHODS AND MATERIALS

Immunizing antigen

F. hepatica were collected from infected sheep livers. The parasites were freed from adhering bile and other contaminating material by washing in running tap water, were suspended in 0.9% NaCl (1 parasite/ml) and homogenized in a top-drive macerator. The crude homogenate was allowed to stand at 0 to 4 C for several hours to allow large particles to settle out. The supernatant fluid was then decanted off and further freed from insoluble

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material by centrifuging at 3000 r.p.m. for 1 hour. This saline extract was usually amber to dark brown in color, and contained about 1 mg protein N/ml.

Alum-precipitated preparations for immunization were made by treating the saline extract with an equal volume of 10% W/V potash alum (Proom, 1943) and adjusting the pH to 6.5 with 5*N* NaOH. The precipitate obtained was washed twice with 0.9% NaCl (containing 1:100,000 merthiolate) and finally suspended in 0.9% NaCl (merthiolated) for injection. Kjeldahl analyses showed that the above procedure precipitated the same amount of protein N as did precipitation with 10% trichloroacetic acid, indicating that the alum preparation contained all the proteins in the saline extract.

Precipitating antigen

In order to obtain a more purified protein material for use as a precipitating antigen in qualitative and quantitative precipitin tests, the crude saline extract was subjected to salt fractionation. A protein fraction was obtained which precipitated at half-saturation with $(NH_4)_2SO_4$, and this material, after several reprecipitations, was used as the precipitating antigen in all our experiments.

The saline extract was treated with 1 volume of saturated $(NH_4)_2SO_4$ solution with stirring, and the mixture was allowed to stand at 0 to 4 C for several hours. The precipitated protein was centrifuged off and treated with 1 volume of 0.1 *M* phosphate buffer, pH 8. Any material which did not redissolve was removed by centrifuging, and the protein fraction was twice reprecipitated with $(NH_4)_2SO_4$ as before. The precipitated material was dialysed against 0.9% NaCl until the dialysate gave no reaction with Nessler's reagent. At each precipitation and during dialysis there was some loss of protein as an insoluble residue, and the yield ultimately obtained was usually about 10% of the total protein in the original saline extract.

Immunization

Rabbits were injected at 8- to 10-day intervals with alum-precipitated antigen prepared as described above. Five ml of a suspension containing 10 mg protein N/ml were injected intramuscularly into each hind leg. Samples of serums were collected 9 to 10 days after each injection and were tested for precipitins with the precipitating antigen. Most of the rabbits gave some reaction after one injection, and all gave a good response after two injections.

Seven rabbits (group A) received a total of 3 injections and a further seven (group B) received a total of 6 injections.

Precipitin tests

Successive small volumes of a stock antigen solution were added to duplicate samples (1.0 ml) of serum, with incubation at 37 C (1 hour) and refrigeration (1 to 2 hours) after each addition of antigen, until no further precipitate was obtained. This was taken to correspond to the complete removal of the corresponding antibody from solution. Each precipitate was centrifuged off, washed twice with 5 drops of ice-cold saline each time to remove nonspecific proteins (Heidelberger and Kendall, 1932), dissolved in a few drops of 0.1 *N* NaOH and 1.0 ml of distilled water, and transferred to micro-Kjeldahl incineration flasks for total N determination.

Control tests were carried out with immune serum plus saline and with antigen solutions plus normal rabbit serum.

Total nitrogen determinations

These were made by the micro-Kjeldahl method, with the NH_3 distilled into a 4% boric acid solution containing a methyl red-methylene blue indicator (see Kabat and Mayer, 1948, p. 283). The final titration was made with *N*/70 HCl, using an "Agla" micrometer syringe (Burroughs Wellcome and Co.) or a microburette, according to the amount of protein N present.

Infection

Each of the immunized and control rabbits was infected with 50 7-day-old cercariae encysted on cellophane and fed in cabbage as described by Urquhart (1954).

RESULTS

Precipitin tests

Qualitative precipitin tests showed that the serums of infected rabbits contained antibodies which reacted with the precipitating antigen prepared from *F. hepatica*. Serums from more than 20 normal rabbits were tested with the precipitating antigen and in no case was any positive reaction obtained.

In order to obtain a measure of the amount of circulating precipitin in the experimental animals and to investigate the serological homogeneity of the precipitating antigen used, quantitative precipitin tests were carried out. The

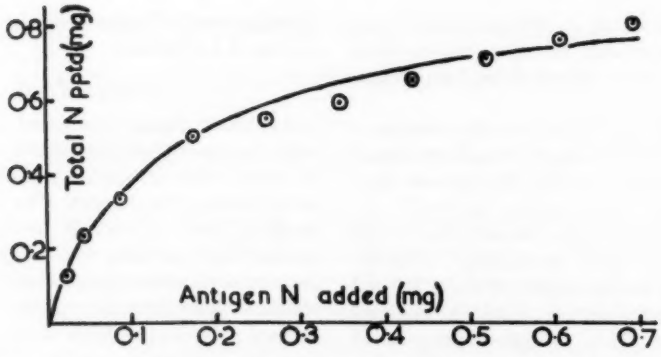

FIG. 1.—Precipitin curve obtained by adding increasing amounts of the protein antigen to 1.0 ml samples of serum from an immunized, infected rabbit.

type of precipitin curve obtained with the antigen and serum from immunized or infected rabbits was characteristic of that shown by inhomogeneous systems (see fig. 1), i.e., excess antigen was demonstrable in the supernatant in the antibody excess zone and there was thus no clearly defined equivalence zone. The region of maximal precipitation was also greatly extended and there was no evidence of inhibition by excess antigen.

In a system of this type it is impossible to determine the antigen and antibody separately in the precipitates unless by some method of labelling one of the reactants, e.g., with a radioactive isotope (Banks, Francis, Mulligan and Wormall, 1951). We considered it likely, however, that the proportion of antibody in the precipitates in the region of maximal precipitation is fairly constant, and thus the figure for total N precipitated in this region probably gives a good measure of the relative amounts of precipitin to the test antigen in the different serums. We have confirmed this by further work with isotopically labelled antibody (see *Discussion.*)

The results of the quantitative tests on the serums of the immunized and control rabbits are shown in table 1.

A course of 3 injections of the alum-precipitated preparation (group A) gave a level of precipitin to the test antigen comparable to that obtained in normal rabbits 63 days after infection with 50 cercariae (control group). In group A the precipitin level 63 days after infection was in most cases considerably greater than the level at the time of infection. This increase was presumably due to the stimulus of infection on the immunized animals. No such increase occurred in group B; here the rabbits were subjected to a course of 6 injections prior to infection, and the precipitin level at the time of infection was considerably higher than in group A.

TABLE 1.—*Quantitative precipitin tests.*

	Rabbit no.	Antibody N plus antigen N precipitated from 1.0 ml serum	
		Pre-infection (mg N)	63 days after infection (mg N)
Immunized rabbits, Series A	V1	0.155	0.774
	V2	0.188	1.868
	N856	0.268	0.620
	N851	0.118	0.636
	0330	0.198	0.498
	0329	0.114	0.150
	0371	0.190	
Immunized rabbits, series B	P93	1.280	1.130
	073	1.170	0.922
	049	0.894	0.768
	084	0.844	0.660
	P947	0.846	0.588
	076	0.620	0.656
	G65	0.420	0.968
Control rabbits,	C2		0.265
	C4		0.095
	C8		0.218
	C9		0.330
	C14		0.238
	X		0.148
	9		0.295
	0		0.138
	Z		0.227
	1		0.117

Autopsies on immunized and control rabbits

The experimental rabbits were killed 63 days after infection and the number of flukes recovered from each of the livers on autopsy are recorded in table 2. In each case the type of lesion found in the liver was noted.

It is evident from the results shown in table 2 that the average number of flukes in each rabbit in the immunized groups is not significantly lower than the average in the control group. However, the flukes recovered from the immunized groups were invariably smaller than those from the control group. This retarded development was particularly marked in the flukes obtained from rabbits V2, V1, 049 and G65. In order to obtain more definite information on this retardation of development, the flukes obtained from group B and from some of the control rabbits were analyzed for total N. The flukes were removed from the liver, rinsed in saline, and analyzed for total N by the micro-Kjeldahl method. The results of these determinations are shown in table 3. The figures obtained confirmed our observations on the inhibited development of the flukes in the immunized rabbits, i.e., the 125 flukes obtained from group B had an average total N content of 0.41 mg, whereas the 108 flukes from 5 control rabbits had an average total N content of 1.13 mg. Examination of the most retarded of these flukes showed that they were sexually immature.

TABLE 2.—*Number of flukes found in immunized and control rabbits on autopsy.*

	Rabbit no.	Number of flukes
Immunized rabbits, group A	V1	10
	V2	3
	N856	6
	N851	10
	0330	13
	0329	18
	0371	26
	Average,	12.3±7.1
Immunized rabbits group B	P93	14
	073	14
	049	3
	084	31
	P947	17
	076	20
	G65	26
	Average,	17.9±9.1
Control rabbits	C2	6
	C4	18
	C8	20
	C9	25
	C14	25
	C7	6
	X	6
	9	25
	0	18
	Z	34
	1	25
	Average,	18.1±9.4

TABLE 3.—*Comparative development of flukes, expressed in terms of their average total nitrogen content, in immunized and control rabbits.*

	Rabbit no.	Number of flukes	Total fluke nitrogen (mg)	Nitrogen per fluke (mg)
Immunized rabbits, group B	P93	14	7.28	0.520
	073	14	5.96	0.426
	049	3	0.56	0.187
	084	31	13.40	0.432
	P947	17	7.54	0.444
	076	20	14.40	0.720
	G65	26	3.18	0.122
			Average,	0.41±0.20
Control rabbits	X	6	4.22	0.704
	9	25	32.5	1.30
	0	18	24.2	1.35
	Z	34	38.8	1.14
	1	25	29.0	1.16
			Average,	1.13±0.25

In general, the livers of the immunized animals showed similar lesions to those of the control group, i.e., moderate fibrinous peritonitis, a degree of fibrosis approximately proportional to the number of flukes present, and a grossly distended common bile duct which usually contained about half of the total number of flukes, the remainder being found in bile ducts in the liver. In rabbits V2, V1, G65 and 049, however, from which the smallest flukes were obtained, there was very extensive fibrinous peritonitis, the livers were very enlarged with numerous large fibrous nodules and showed extensive areas of recent necrosis, caused by damage to blood vessels by migrating flukes. In V2 and O49 the common bile duct was normal in size and contained no flukes, while in V1 and G65 there was a slightly enlarged common bile duct containing a small number of flukes. The

majority of the flukes from these 4 rabbits were found either migrating through liver tissue or in very fine bile ducts.

DISCUSSION

The immunological relationship between a helminth parasite and its host is likely to be complicated. The worm itself is a complex organism and is certain to contain quite a number of antigenic compounds and complexes. Some of these antigens will be tissue or structural components of the parasite; others may be enzymes or products of its metabolic processes. The presence of these antigens may be demonstrated by using extracts of the parasite in serological tests on infected animals or by using similar extracts as immunizing antigens. Difficulties are likely to arise in the attempted integration of the results from these two approaches, e.g., the parasite may contain a number of antigenic compounds which, because of their location, are never effective in the natural infection and, furthermore, antigens which are functional in the normal infection may be destroyed or altered by the processes involved in extracting them from the parasite.

There is now an adequate amount of evidence to demonstrate that an acquired immunity does occur in a number of helminth diseases (see introduction). This acquired immunity has been demonstrated by a resistance to superinfection or to reinfection after the removal of a primary infection, or by the protective action of extracts of the parasite when used as immunizing antigens. It has usually been possible in these cases to demonstrate by serological tests the presence of circulating antibodies. There has, however, been little work carried out on helminths, comparable to that on bacteria, on the isolation and purification of the antigenic compounds of the organisms, on a study of their immunological activity and specificity, and on the protective power of the antibodies which they elicit. In this connection it should be noted that there is not necessarily any correlation between the capacity of a helminth antigen to elicit the production of antibodies and the protective power of these antibodies; e.g., Campbell (1939) obtained a purified polysaccharide antigen from the larval form of *Taenia taeniaeformis* which stimulated the formation of precipitins but which had no protective action against the disease.

From the present investigation, designed largely to study the antigenic properties of proteins from *F. hepatica* and the protective properties, if any, of the antibodies which they elicit, we have concluded that prior immunization with fluke proteins has no influence on the number of *F. hepatica* developing from a challenging infection. The infection of normal rabbits with 50 7-day-old cercariae has been shown to yield an average "take" of 18.6 ± 8.8. This average is apparently not influenced by the sex of the rabbit nor by the use of cercariae from different snails (Urquhart, 1954). In the experiments described here the control rabbits showed a take of 18.1 ± 9.4, while the immunized groups showed takes of 12.3 ± 7.1 (group A) and 17.9 ± 9.1 (group B), respectively. The reduction in take in group A as compared to that of the control group is not statistically significant. Kerr and Petkovitch (1935) reported that it seemed possible to establish an active immunity in rabbits to *F. hepatica* by injections of dried fluke material. In their experiments 7 immunized rabbits, each infected with 13 cercariae, yielded 1, 2, 2, 2, 2, 3 and 3 flukes, respectively, at autopsy. Several of these flukes were described as "calcified," and repeated fecal examinations failed to demon-

strate the presence of fluke eggs. Three control rabbits (also infected with 13 cercariae) had 11, 5 and 9 flukes at autopsy. None of these flukes was calcified and eggs were found in the faeces of all the rabbits. It is difficult to conclude from this experiment how significant is the apparent reduction in take of the immunized group as compared to controls, because of the small number of rabbits (3) in the control group, and the variation in take which occurs in normal rabbits. If this reduction is real it might be due to the influence of antigens other than the proteins.

In our experiment there is no correlation in either the immunized or normal rabbits between the number of flukes found in a rabbit and the level of circulating precipitin to the protein antigen, as shown by the quantitative tests. It must be noted that in the precipitin tests we have measured the level of antibody to only one protein fraction of the parasite and have expressed our results in terms of the total N (antibody N+ antigen N) precipitated. The chief difficulty in applying standard immunochemical techniques to the quantitative determination of antibody precipitated by relatively crude antigenic fractions is that one cannot assume complete precipitation of the antigen in regions of antibody excess as is possible with a

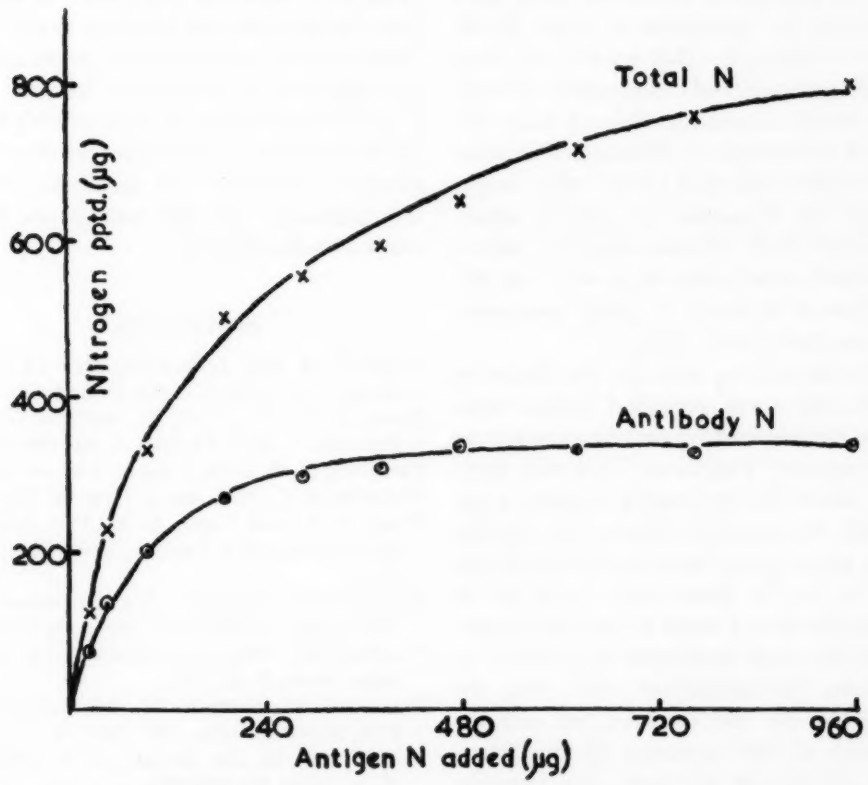

FIG. 2.—Total N and antibody N precipitated from 1.0 ml samples of antiserum by increasing amounts of the protein antigen.

The serum was trace-labelled with I^{131} as described by Francis, Mulligan and Wormall (1951). The specific precipitates were analyzed for total N and radioactivity, and the antibody N calculated by comparing the radioactivity of the precipitates with that of a γ globulin standard prepared from the labelled serum, and analyzed for protein N.

"pure" protein antigen and its homologous antiserum. We have more recently been able to use antiserums labelled with radioactive iodine and thus to determine the absolute amount of antibody N precipitated from a serum by a particular antigenic fraction (see fig. 2). This isotopic technique greatly simplifies and extends the scope of such analyses and will be reported in detail later.

In the immunized groups there was evidence of inhibited development of the parasites, viz., the smaller size of the flukes (confirmed in group B by micro-Kjeldahl analysis), the sexual immaturity of some of these, and the presence of migratory forms 63 days after infection (in autopsies of over 70 infected rabbits at this stage, we have never encountered migratory forms). This result is comparable to that obtained by Schwartz, Alicata and Lucker (1931) and Chandler (1932) who, working on the resistance of rats to superinfection with *Nippostrongylus muris*, observed retardation of growth and development without, in most instances, any marked lethal effect.

It is interesting that the four livers in which the most retarded flukes were found also showed by far the most severe inflammatory reactions. This may have been due to the prolonged migratory period of the retarded flukes, but, on the other hand, there were only three flukes each in two of these livers (table 3). It is possible that a state of specific hypersensitivity had developed as a result of previous immunization and was responsible for the exaggerated cellular reaction to the presence of the parasites. If this is the case, the possible effect of hypersensitivity should be borne in mind in any attempts to control parasitic diseases by means of worm antigens. Similar phenomena have been observed in the study of natural acquired immunity to oesophagostomiasis in sheep (Fourie, 1936; Monning, 1949), to *N. muris* infections in rats (Sarles and Taliaferro, 1936; Taliaferro and Sarles, 1939), to anclystomiasis of mice and ascariasis of guinea pigs (Kerr, 1936 and 1938) and to *Cooperia* infections in sheep (Andrews, 1939).

SUMMARY

1. Serums from rabbits infected with *Fasciola hepatica* were found to contain precipitins which reacted with a protein fraction prepared from the adult parasite.

2. Immunization of rabbits with proteins of *F. hepatica* was shown to stimulate the production of these precipitins in relatively large amounts, as measured by quantitative precipitin tests.

3. Immunization of rabbits with these proteins prior to infection was shown to produce inhibition of development of the parasites, but did not reduce their numbers significantly.

REFERENCES

Africa, C. M. 1931, J. Parasitol. **18**: 1–13.
Andrews, J. S. 1939, J. Agric. Res. **58**: 771–785.
Banks, T. E., Francis, G. E., Mulligan, W. and Wormall, A. 1951, Biochem. J. **48**: 180–187.
Campbell, D. H. 1939, J. Infect. Dis. **65**: 12–15.
Chandler, A. C. 1932, Am. J. Hyg. **16**: 750–782.
Craig, C. F. and Faust, E. C. 1951, Clinical Parasitology, ed. 5, London, Henry Kimpton, p. 21.
Fairley, N. H., Mackie, F. P. and Jasudasan, F. 1930, Indian M. Research Mem. **17**: 53–59.
Fourie, P. J. J. 1936, Onderstepoort J. Vet. Sc. & Anim. Indus. **7**: 277–347.
Francis, G. E., Mulligan, W. and Wormall, A. 1951, Nature, London, **167**: 748–758.
Heidelberger, M. and Kendall, F. E. 1932, J. Exper. Med. **55**: 555–561.
Kabat, E. A. and Mayer, M. M. 1948, Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas.
Kagan, I. G. and Lee, C. L. 1953, J. Infect. Dis. **92**: 52–57.
Kerr, K. B. 1936, Am. J. Hyg. **24**: 381–406.

Kerr, K. B. 1938, Am. J. Hyg. **27**: 28–51.
Kerr, K. B. and Petkovitch, O. L. 1935, J. Parasitol. **21**: 319–320.
Monnig, H. O. 1949, Veterinary Helminthology and Entomology, ed. 3, London, Bailliere, Tindall and Cox, p. 176.
Miller, H. M., Jr. 1932, J. Prev. Med. **32**: 37–46.
Ozawa, M. 1930, Jap. J. Exper. Med. **8**: 79–84.
Proom, H. 1943, J. Path. Bact. **55**: 419–426.
Sarles, M. P. and Taliaferro, W. H. 1936, J. Infect. Dis. **59**: 207–220.
Schwartz, B., Alicata, J. E. and Lucker, J. T. 1931, J. Wash. Acad. Sc. **21**: 259–261.
Stewart, D. F. 1950, Australian J. Agric. Res. **1**: 301–321.
Stoll, N. R. 1942, J. Parasitol. suppl. **28**: 20.
Taliaferro, W. H. 1940, Physiol. Rev. **20**: 469–492.
Taliaferro, W. H. and Sarles, M. P. 1939, J. Infect. Dis. **64**: 157–192.
Urquhart, G. M. 1954, Exper. Parasitol. In press.
Watts, N. P. 1949, J. Immunol. **62**: 183–192.

THE PROVIDENCE GROUP: AN INTERMEDIATE GROUP OF ENTERIC BACTERIA

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In 1943 Stuart and his collaborators described a group of paracolon bacteria which they designated type 29911. A more detailed study of such cultures was reported by Stuart et al in 1946. In the latter publication, the general bacteriological characteristics and biochemical reactions of 29911 cultures were defined. Brooke (1951) reported on the biochemical and cultural characteristics of a number of 29911 paracolon bacteria isolated from urine specimens and employed the term Providence group, a designation that was proposed by Kauffmann (1951) for these cultures.

The Providence group may be defined as follows: Gram-negative bacteria that conform to the definition of the family Enterobacteriaceae. Acid is produced from glucose within 24 hours and the majority of cultures form a small amount of gas. Lactose is not fermented. Most cultures utilize sucrose upon prolonged incubation while mannitol usually is not fermented. Adonitol is utilized by the majority of cultures. Indol is produced, growth occurs on Simmons' citrate agar, and the methyl red reaction is positive. Acetylmethylcarbinol is not formed, urea is not rapidly decomposed, and hydrogen sulfide is not formed in Kligler's agar or triple sugar iron agar. With very few exceptions, the bacteria are motile.

In 1949 Dr. Stuart kindly furnished 114 cultures of 29911 paracolon bacteria and several antiserums which he and his associates employed in their studies. These strains, together with a number collected by the senior author in Italy during 1944 and 1945, became the nucleus of the present collection of Providence group cultures. Since then, a very large number of paracolon bacteria from nearly all states of the Union and from many widely scattered foreign countries were collected and examined. From this large accumulation of paracolon strains, 631 cultures were selected that gave biochemical reactions by which they could be classified as members of the Providence group. The sources of 543 of these cultures that were examined serologically were as follows:

Feces	185
Urine	15
Other localizations	10
Not known	333

Of the cultures from feces, 37 were from cases of diarrhea, 123 were from food handlers, 1 was from a dog, and histories were not obtained with the remaining 24. The cultures from other localizations were as follows: blood cultures, 2, colon at autopsy, 2, suprapubic abscess, 2, prostatic fluid, 1, pericardium at autopsy, 1, autopsy tissue of a cat, 1, and irrigation waters, 1. Since the majority of the cultures were received from state, county, and city departments of health and from hospital laboratories, there can be little doubt that most of the cultures were of human origin and that they were isolated from stools of persons affected with diarrhea and from urine in cases of urinary tract infection.

Since there was little correlation between the sources of the cultures and the O antigen group to which they belonged,

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these data were not included. However, it should be noted that 5 of the cultures from urine belonged to O antigen group 4, and 7 belonged to O group 23.

Several cultures that previously were described as shigellas were included in the group; there were types B81 and B105 (Sachs, 1943), type P25 (MacLennan, 1945), and *Bacterium wakefield*, which was described by Berger (1945). Data on the prevalence of these types are given in the papers cited.

RESULTS

General bacteriology and biochemical reactions

Members of the Providence group form colonies similar to those of other enteric bacteria on plain infusion agar plates. Variation in colony form from opaque to translucent forms (KO to O forms) is common. Also, smooth to rough (S-R) variation occurs particularly in cultures maintained in the laboratory for some time. Members of the group grow well on eosin methylene blue, Salmonella-Shigella, and MacConkey's agars and the colonies present appearances similar to those of salmonellas or shigellas. In contrast to shigellas, Providence group cultures grow well on bismuth sulfite (Wilson-Blair) agar. On this medium, colonies may be colorless, light green, or olive green, and colonies of some cultures may be slightly blackened with the occasional production of metallic sheen. Of 100 strains tested on brilliant green agar, only one grew well and produced alkalinization of the medium. In Kligler's iron agar or triple sugar iron agar (TSI), the reactions simulated those of shigellas, except that evidence of a small amount of gas production may be apparent in the butt of the tube. Hydrogen sulfide is not formed nor is lactose utilized in these mediums; hence, there is a shigella-like appearance. Although sucrose is utilized by most Providence group cultures, fermentation usually is delayed and therefore not apparent in TSI agar.

The 631 cultures were tested for indol production, citrate utilization, the methyl red, and Voges-Proskauer reactions, for urea hydrolysis on Christensen's medium, for nitrate reduction, motility, and for utilization of the carbohydrates listed in table 1. Since 20 of the cultures were not tested for adonitol and inositol fermentation, they were not recorded in table 1. All cultures produced indol and were methyl red-positive. Simmons' citrate agar was alkalinized by 630 of the strains, the reaction usually becoming apparent within 24 hours. Acetylmethylcarbinol was not produced. Nitrates were reduced to nitrites. Rapid decomposition of urea did not occur, but three cultures produced weak, delayed reactions. Providence group cultures usually were motile but it should be emphasized that motility often was very sluggish.

It was possible to separate the cultures into two main biochemical groups. This separation was based primarily upon gas production from glucose and upon reactions obtained in mediums that contained adonitol and inositol (table 1). Biochemical group 1 cultures usually formed a bubble to about 15% of gas from glucose, usually fermented adonitol, and rarely utilized inositol. In contrast, biochemical group 2 cultures were anaerogenic, fermented inositol, and usually did not produce acid from adonitol. Admittedly, this division was based upon arbitrary criteria to which exceptions could be found. However, it should be noted (table 1) that biotypes 1, 2, and 3 composed 86% of the biochemical group 1 strains, that biotypes 21, 22, and 23 made up 80% of the biochemical group 2 cultures, and that the distinction between biochemical group 1 and biochemical group 2 was quite clear

TABLE 1.—*The biochemical reactions of 611 Providence group cultures.*

Biochemical group	Biotype	Number of cultures		Percent Biogroup	Percent total	Glucose	Lactose	Sucrose	Mannitol	Adonitol	Salicin	Inositol
		Biogroup 1	Biogroup 2									
1	1	342		65.		Ag	—	(A)	—	Ag	—	—
	2	62		11.5		Ag	—	A	—	Ag	—	—
	3	52		10.		Ag	—	—	—	Ag	—	—
	4	16		3.2		A	—	(A)	—	A	—	—
	5	10		1.9		Ag	—	(A)	—	—	—	—
	6	10		1.9		A	—	(A)	—	—	—	—
	7	9		1.7		A	—	—	—	A	—	—
	8	5		.95		Ag	—	—	—	—	—	—
	9	3		.5		A	—	—	—	—	—	—
	10	3		.5		Ag	—	Ag	Ag	Ag	—	—
	11	3		.5		Ag	—	—	Ag	Ag	—	—
	12	2		.35		Ag	—	(A)	—	Ag	—	Ag
	13	1		.19		A	—	(A)	A	—	—	—
	14	1		.19		Ag	—	—	A	A	A	—
	15	2		.35		Ag	—	—	Ag	—	—	—
	16	2		.35		A	—	A	—	A	—	—
	17	1		.19		Ag	—	—	Ag	Ag	—	Ag
	18	1		.19		Ag	—	Ag	—	Ag	Ag	Ag
	19	2		.35		Ag	—	Ag	Ag	—	Ag	—
	20	1		.19		A	—	(A)	(A)	(A)	—	—
		528			86.5							
2	21		51	61.5		A	—	(A)	—	—	—	A
	22		11	13.3		A	—	A	—	—	—	A
	23		5	6.		A	—	—	—	—	—	A
	24		2	2.4		A	—	(A)	A	—	—	A
	25		2	2.4		A	—	(A)	—	A	—	A
	26		4	4.8		A	—	A	A	—	—	A
	27		1	1.2		A	—	(A)	(A)	—	—	A
	28		1	1.2		A	—	(A)	—	—	—	(A)
	29		3	3.6		A	—	A	—	—	—	(A)
	30		2	2.4		A	—	(A)	A	A	—	A
	31		1	1.2		A	—	A	(A)	—	—	A
			83		13.5							
Total		611			100							

A, Ag, acid or acid and gas.
—, No reaction in 30 days.
( ), Reaction delayed 48 hours or longer.

in these instances. Biotypes 1, 2, 3, 21, 22, and 23 comprised 83% of the 611 strains on which complete biochemical tests were made. Cultures of biochemical group 1 predominated in all except 7 of the O antigen groups. The majority of cultures of O antigen groups 4, 20, 43, 44 and 47 were members of biochemical group 2, and in O antigen groups 8 and 23 the division was nearly equal.

One hundred Providence group cultures also were tested in several mediums, in addition to those mentioned above or listed in table 1. Representatives of each O antigen group were included in this group of strains. The strains tested did not produce acid from dulcitol. Both arabinose and xylose were utilized by only one culture, a strain that belonged to biotype 4. Xylose was utilized by one additional culture (biotype 25). All other cultures tested did not ferment xylose or arabinose. Trehalose usually was fermented by biochemical group 2 strains, while very few biochemical group 1 cultures utilized this substrate. Biochemical group 2 strains were variable in their utilization of sorbitol, while none of the biochemical group 1 cultures tested fermented this substance. Biochemical group 1 cultures did not liquefy gelatin during the 30-day observation period, but 5 biochemical group 2 strains produced slight liquefaction after 3 weeks incubation.

Serological methods

The methods employed in the investigation of the serological properties of the Providence group cultures were based upon those recommended for the study of the *Escherichia coli* group (Kauffmann, 1947, 1951). Antiserums

for the O antigens were prepared by injection of broth cultures that were heated at 100 C for 2½ hours when smooth translucent forms (O or K minus) were available. When it was necessary to employ the more opaque (KO or K plus) colony forms, the broth cultures were heated at 121 C for 2 hours. In either case, young broth cultures that were incubated for 6 to 8 hours proved to be the most satisfactory for O antiserum production. Broth cultures that were heated at 100 C for 1 hour or, when necessary, at 121 C for 2 hours, were employed in tube agglutination tests which were incubated at 50 C for 16 to 18 hours to determine O antigens. Thick suspensions used in slide agglutination tests were prepared in 0.5% saline solution and were heated at 100 C or 121 C.

Broth cultures used for the production of antiserums for flagellar (H) components and for antigens in H agglutination tests were incubated for 15 to 18 hours and then were preserved by the addition of 0.5% formalin. As mentioned above, most Providence group cultures were sluggishly motile when first isolated and were not flocculated rapidly in the manner characteristic of flagellar agglutination. In order to obtain suitable H antigens it was necessary first to promote motility by passage of the cultures through semisolid medium. A medium that contained 0.3% agar was employed for the first 3 or 4 passages and a medium with 0.4% agar was used for subsequent transfers. Usually 6 or 7 passages were necessary before satisfactory antigens were obtained for use in antiserum production and for H agglutination tests. However, some strains required 10 or more passages. Tests for H agglutination in unabsorbed antiserums were incubated in a water bath at 48 to 50 C and reactions were read at the end of ½ and 2 hours. When absorbed H antiserums were used, the tests were read after ½, 2, and 4 hours' incubation. A complete set of H antiserums from which the O agglutinin was removed was prepared by treating each antiserum with a suspension of the homologous strain after it was heated at 100 C for 1 hour.

Only a few antiserums for the detection of envelope or K antigens were prepared. Cultures to be used for K antiserum production were plated on infusion agar and smooth opaque colony forms were selected for transfer to infusion broth. Such broth cultures were incubated at 37 C for 4 to 6 hours and then injected into rabbits. The broth cultures used for the first injection were killed by the addition of 0.5% formalin. Subsequent inoculations were made with living cultures. The suspensions employed in K agglutination tests were similar to those used for antiserum production. These tests were placed first in an incubator at 37 C for 2 hours and then removed to a refrigerator for about 16 hours.

The heat stable somatic (O) antigens

In all, 85 O antiserums were prepared with Providence group cultures. With these antiserums it was possible to establish 56 O antigen groups. Of the total of 631 cultures, 543 were smooth and in suitable condition for serologic examination. It was possible to classify 522 or 96% of the smooth cultures in one or another of the 56 O antigen groups.

In the establishment of the 56 O antigen groups, cultures that exhibited reciprocal relationships were placed in the same O antigen group. Cross agglutination that amounted to 10% or more of the titer of the antiserums was accepted as an arbitrary criterion for such grouping. Cultures that bore only slight relationships to each other, or which reacted unilaterally, were placed

TABLE 2.—*The O antigenic relationships of Providence group cultures.*

O antiserums	Homologous titer	Titer with other O antigen groups*
1	5,120†	*3* (320), *21* (320)
2	5,120	
3	10,240	*1* (320), *21* (320)
4	2,560	
5	20,480	
6	5,120	15 (320), 41 (640)
7	20,480	10 (160), 15 (320)
8	20,480	
9	10,240	
10	2,560	13 (320), 15 (160)
11	20,480	
12	5,120	
13	2,560	
14	20,480	
15	20,480	
16	20,480	
17	5,120	
18	5,120	15 (160), 37 (640)
19	2,560	15 (640)
20	2,560	
21	5,120	*1* (320), *3* (640)
22	20,480	
23	10,240	
24	2,560	
25	20,480	14 (320)
26	20,480	
27	20,480	
28	5,120	
29	2,560	32 (80)
30	20,480	11 (640)
31	20,480	
32	10,240	
33	5,120	
34	10,240	
35	2,560	
36	5,120	30 (320)
37	20,480	42 (80)
38	5,120	32 (1280)
39	2,560	
40	10,240	34 (320)
41	20,480	11 (1280)
42	20,480	
43	20,480	
44	5,120	34 (1280)
45	10,240	42 (320)
46	5,120	
47	5,120	
48	10,240	
49	2,560	
50	5,120	10 (640)
51	2,560	
52	2,560	
53	10,240	18 (160)
54	2,560	
55	2,560	
56	10,240	

* Reciprocal relationships are italicized.
† Figures are reciprocal of highest dilution that gave strong agglutination.

in separate O antigen groups. It is obvious that the O antigens of all cultures contained in any one O group were not necessarily identical. The reactions obtained with each of the 56 O antigen groups are recorded in table 2.

It was possible to divide O antigen groups 5, 8, 15, 23, 37, 45, and 49 into a,b and a,c subgroups. In these, cultures were found that shared an O antigen fraction, labeled a, but some of them contained a second factor, b, while others possessed a third factor, c. Factors b and c were not shared. As an example, it was possible to classify some O group 8 cultures as 8a, 8b and others as 8a, 8c.

Of the 543 cultures, 15.5% belonged to O antigen group 3. Members of O groups 23 (5.5%), 9 (4.5%), 21 (4.5%), 4 (4%), and 19 (4%) also were found often. These six O antigen groups accounted for about 40% of the strains.

The flagellar (H) antigens

In all, 54 antiserums were prepared for the flagellar components of Providence group cultures. With these antiserums it was possible to designate 28 distinct H antigens, the raections of which are given in table 3. The individual H antigens of Providence group cultures showed little relationship one to another. Certain H antigens occurred in cultures of several different O antigen groups (table 4). For example, H antigen 1 occurred in cultures that belonged to 12 different O antigen groups and H 4 occurred in 17.

The reactions of the H antigens were confirmed by tests in antiserums from

TABLE 3.—*The H antigens of Providence group cultures.*

H antiserums	Homologous titer	Titers with other H antigens
1	12,800	25 (200)
2	25,600	3 (3200)
3	6,400	
4	6,400	*20* (100) *2* (400)
5	25,600	
6	6,400	
7	12,800	
8	12,800	23 (400)
9	12,800	
10	6,400	
11	12,800	
12	25,600	
13	25,600	8 (1600)
14	25,600	
15	12,800	
16	6,400	
17	6,400	
18	12,800	
19	12,800	
20	12,800	*4* (200) *28* (800)
21	25,600	
22	25,600	
23	12,800	*7* (400)
24	25,600	1 (800)
25	6,400	
26	25,600	
27	25,600	
28	25,600	*4* (400) *20* (800)

See footnotes, tables 1 and 2.

TABLE 4.—*Providence group antigenic schema (provisional).*

O group	H antigens that occur in O group
1	1(7), 3(1), 11(1), NM(1), ND(1)
2	2(11), ND(1)
3	3(45), 11(12), 18(1), 22(1), NM(1), ND(17), U(2)
4	4(14), 14(1), NM(3), ND(4)
5	1(1), 4(1), 5(1), 16(10), U(3), ND(1)
6	6(11), 18(1), ND(2)
7	7(2), ND(1)
8	4(1), 17(1), 19(1), 23(1), 27(1)
9	1(1), 8(3), 11(13), 16(1), 21(1), 25(1), U(1), ND(1)
10	9(1), 28(1), NM(3)
11	10(1), 16(1)
12	3(1), 15(5)
13	11(2), 18(1)
14*	4(1), 12(1), NM(3), U(2)
15*	NM(1)
16	3(1), 17(1), 24(3), 25(1), U(1), ND(3), NM(1)
17	3(2), 7(1), 11(1), 15(1), 23(1), ND(1)
18	14(1)
19	2(9), U(1), ND(12)
20	4(1), ND(1), NM(1)
21	4(1), 11(4), 23(9), ND(10)
22	1(6), 2(4), 3(3), U(1), ND(4)
23	1(1), 3(1),4 (11), 13(2), 20(1), U(2), NM(1), ND(7)
24	4(1), 8(1), 15(1), ND(4),
25	4(1)
26	1(2), 11(3), 28(1), U(1), ND(4)
27	8(1), 17(13), ND(2)
28	9(2), 18(6), ND(2)
29	2(3), 3(1), NM(2)
30	3(2), 18(2), 19(5), U(2), ND(3)
31	3(2), 7(1), 11(1), 17(1), 18(1), 23(1), ND(2)
32	3(1), 11(3), U(1)
33	1(2), 23(1)
34	18(1), U(4)
35	18(1)
36	1(3), 18(1), 19(1), ND(1)
37	1(9), 4(1), U(3), ND(4)
38	1(2), 18(1), 22(1), U(1), NM(1), ND(1)
39	18(5), ND(1)
40	23(1)
41	18(3), ND(5)
42	15(2), ND(2)
43	4(5), 28(1), ND(1)
44	4(1), ND(1)
45	11(1), 22(1), 25(1), 26(1), U(5), NM(2)
46*	1(1), 18(3), ND(2)
47	4(1)
48	25(1)
49	1(3)
50	3(2), U(2)
51	15(1), 25(1), ND(1)
52	4(2)
53	2(1), U(4), ND(2)
54	4(1), U(1)
55	4(1), 11(1)
56	4(1)

( ), Figures in parentheses indicate the number of strains of each type.
U, Undetermined; H antigen did not belong to any of the 28 known H antigens.
NM, Nonmotile.
ND, H not done.
* K antigen 1 (α antigen of Stamp and Stone) occurs in O groups 14, 15, and 46. K antigen 2 occurs in O group 25.

which O agglutinins were removed by absorption with heated cultures.

Several attempts were made to determine whether or not antibody for the heat labile somatic (K) antigens might be present in any of the H antiserums. When possible, a nonmotile variant of the homologous strain was tested and when this was not possible, poorly motile colonies were selected for the tests. None of the tests revealed the presence of K antibody in the H antiserums. In the few instances where reactions occurred, they were weak and of low titer and were attributable to O or to H agglutination. Typical K agglutination was not seen. It seemed probable that the K antigens of the cultures had been lost in the course of the many passages through semisolid medium that were required to produce satisfactory suspensions for H antiserum production. In any event, it was clear that the H antigens could be determined easily if satisfactory antigens and antiserums were available.

The heat labile somatic (K) antigens

There was no doubt that Providence group cultures contain K antigens since the cultures were either inagglutinable or poorly agglutinable in O antiserums when tested in the living state. Also, dissociation into translucent and opaque colony forms could be demonstrated in the majority of cultures. This variation was independent of smooth to rough variation. One culture, belonging to O group 25, possessed a distinct capsule. An antiserum prepared with a living suspension of the encapsulated strain gave typical capsular agglutination in tube tests and gave positive Quellung reactions. Absorption tests indicated that the capsular antigen of this strain was an L antigen (Kauffmann, 1951). This thermolabile antigen was designated K antigen 2 of the Providence group (table 4).

The α antigen of Stamp and Stone (1944) occurred in some cultures of O groups 14, 15, and 46. This antigen should be considered as one of the K antigens of the group. However, α antigen did not occur frequently in Providence group cultures. Of 473 strains tested in three α antiserums, only 4

possessed α antigen. Two of these belonged to O group 14, one to O group 15, and one to O group 46. These cultures were received some years ago and represent the original α antigen cultures. Living suspensions of cultures known to contain α antigen were tested in all of the O and H antiserums prepared with Providence group cultures. Alpha antibody was not detected in any antiserum.

Antigenic schema for the Providence group

The studies mentioned above on the O and H antigens of Providence group cultures made it possible to devise an antigenic schema for the group. The schema proposed in table 4 consists of 56 O antigen groups, 28 H antigens, 2 K antigens and 125 serological types.

DISCUSSION

The Providence group appears to be an intermediate group of enteric bacteria that resemble *Proteus morgani* and *Proteus rettgeri* in many ways but which differ from these *Proteus* species in certain important aspects, such as failure to hydrolyze urea. Members of the group occur commonly in stools and in urinary tract infections and often are mistaken for shigellas. They differ from the latter in that they utilize citrate, are motile, and usually ferment either adonitol or inositol.

When the biochemical reactions of a culture indicate that it is a member of the Providence group, serologic methods then may be used to determine its type. Slide agglutination tests with pooled O group antiserums or with specially prepared grouping antiserums may be employed for preliminary examination of cultures. Early in the study, pools composed of five O group antiserums each were used for grouping purposes. Later, grouping antiserums were prepared in animals by injection of mixed vaccines each consisting of ten O group cultures. Following examination in the grouping antiserums, the O group of a culture may be determined by examination in the separate O group antiserums. This may be done by slide agglutination tests if the results of such tests are confirmed by single tube agglutination tests using a previously determined diagnostic dilution of antiserum or by titration.

Bacteria of the group have been isolated from stools in sporadic cases and small outbreaks of diarrhea in man, but they occur also in the feces of normal individuals. It is hoped that typing methods will aid in the collection of data which will help determine the pathogenic propensities of Providence group cultures.

SUMMARY

A biochemical and serological study was made of 631 Providence group cultures. Fifty-six O antigen groups were established and 28 flagellar antigens were designated. An antigenic schema for the group was devised which contained 125 serological types.

REFERENCES

Berger, F. M. 1945, J. Hyg. **44**: 116–119.

Brooke, M. S. 1951, Acta path. et microbiol. Scandinav. **29**: 1–8.

Kauffmann, F. 1947, J. Immunol. **57**: 71–100.

———. 1951, Enterobacteriaceae, Copenhagen, Einar Munksgaard.

MacLennan, J. D. 1945, J. Path. Bact. **57**: 307–315.

Sachs, A. 1943, J. Roy. Army Med. Corps, **80**: 92–99.

Stamp, L., and Stone, D. M. 1944, J. Hyg. **43**: 266–272.

Stuart, C. A., Wheeler, K. M. and McGann, V. 1946, J. Bact. **52**: 431–438.

Stuart, C. A., Wheeler, K. M., Rustigian, R. and Zimmerman, A. 1943, J. Bact. **45**: 101–119.

A PREFERRED METHOD FOR THE ROUTINE IDENTIFICATION OF CANDIDA

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It has been suggested that species of candida isolated from patients can be differentiated either by fermentation reactions or by differences in morphology. However, reports by a number of workers[1–13] concerning the consistency of the results obtained with both methods are conflicting.

During the last two years, an extensive series of comparative tests were carried out to determine which was the more consistent routine identification of candida species. The experiments involved hundreds of strains and hundreds of reactions. The results of these tests showed that fermentation reactions were inconsistent. In fact, the fermentation reactions of cultures of *Candida albicans* isolated even from the same patient were, except in their ability to ferment glucose, equally inconsistent. In addition, progeny from single-cell cultures of *C. albicans* differed in fermentation ability not only from the parent culture but also among themselves.

On the other hand, when differences in morphology were used, candida species were identified more rapidly and with greater consistency. *C. albicans* was identified in 1 to 3 days from other candida species by its ability to form characteristic chlamydospores on yellow corn-meal agar. Though some variations occurred in the time required for different isolations of *C. albicans* to form chlamydospores, the results of these tests showed that the identification of candida species based on morphological differences is more rapid and consistent than fermentation reaction.

The following procedure has been

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1. Benedek, T. 1943, Are fermentation tests and biochemical characteristics reliable in the differentiation of monilias? Mycopath. 111, 3–4: 346–353.
2. Langeron, M. and Talice, R. 1932, Nouvelles methodes d'etude et assai de classification des champignons levuriformes. Ann. Parasit. et Compar. **10**: 1–80.
3. Martin, D. S., Jones, C. P., Yao, K. F. and Lee, L. E. 1937, A practical classification of the monilias. J. Bact. **34**: 99–128.
4. Martin, D. S. and Jones, C. P. 1940, Further studies on the practical classification of the monilias. J. Bact. **39**: 609–630.
5. Raubitschek, F. 1945, A new fermentation method for mycological identification. J. Bact. **3**: 337–339.
6. Skinner, C. E. 1947, The yeast-like fungi: Candida and Brettanomyces. Bact. Rev. **11**: 227–274.
7. Wachiowak, M., Marr, J., Hagebusch, O. E., Randall, W. A. and Fleischer, M. S. 1934, Differentiation of various strains of Monilia by cultural methods. J. Infect. Dis. **54**: 35–44.
8. Wickerham, L. J. 1946, A critical evaluation of nitrogen assimilation tests commonly used in the classification of yeasts. J. Bact. **3**: 293–301.
9. Benham, R. W. 1931, Certain monilias parasitic on man. Their identification by morphology and by agglutination, J. Infect. Dis. **49**: 183–215.
10. Kligman, A. M. 1950, Aids in technic in the identification of *Candida albicans*. J. Invest. Dermat. **14**: 173–176.
11. Ajello, L. 1945, A simple method for preparing corn meal agar. Mycologia, **37**: 636.
12. Bernhardt, E. 1946, Time saving in the preparation of corn meal agar and in the identification of yeast-like fungi, Mycologia, **38**: 228–229.
13. Gordon, M. A., Bradley, G. E. and Grant, V. Q. 1952, The influence of different types of corn meal agar upon chlamydospore production by *Candida albicans*. J. Lab. & Clin. Med. **40**: 316–320.

used, therefore, in this laboratory and is recommended as a preferred method for the routine identification of candida species.

MATERIALS AND METHODS

1. Detection.—Specimens suspected of moniliasis are examined directly, either as stained smears or as potassium hydroxide mounts.

A methylene blue, basic fuchsin stain devised by Mr. William Carmichael and modified by myself is employed in this laboratory. It is prepared by adding 2 cc of a saturated alcoholic solution of methylene blue (1.48%) and 4 cc of a saturated alcoholic solution of basic fuchsin (0.66%) to 27 cc distilled water. This stain should be made freshly every week. Fixed smears are immersed in this stain for 1 to 2 minutes, washed in running water, and dried in air. Tissue sections on slides may also be stained in this manner. Yeast cells and hyphal elements appear dark blue, while epithelial cells and purulent material remain pink.

Small, spherical to oval budding cells 1.5 to 5.0 μ and hyphal elements suggest candida; larger cells which do not bud, some of which are rectangular with rounded ends, 3 to 3.5 by 6.5 to 7.5 μ, and others spherical, 5.3 to 8.7 μ, suggest *Geotrichum.*

2. Inoculation.—Specimens are inoculated on Sabouraud's glucose agar (modified by the substitution of phytone for peptone and the addition of yeast extract) and more heavily on Littman oxgall agar.[14] Pure cultures of fungi causing "double infections" have been isolated more easily from the Littman medium. For example, skin scrapings from patient #3168 cultured on Littman oxgall agar yielded discrete colonies of *C. albicans* and *Trichophyton rubrum*, while plates of Sabouraud's glucose agar inoculated with similar material were too contaminated with staphylococci and penicillium for satisfactory examination.

The inoculated plates are incubated at room temperature (22 to 24 C) and examined after 24 hours. Colonies of candida species on Sabouraud's glucose agar resemble the descriptions outlined by Martin et al.[3] On Littman agar they are smooth, shiny, convex, entire and of varying shades of blue.

3. Identification of candida species.—Species of candida are differentiated routinely from other "yeasts" by their ability to form characteristic pseudomycelium on a nutrient-poor medium such as corn-meal agar. The pathogenic *C. albicans* can be identified rapidly since it is the only candida able to form chlamydospores on this medium.

It has been noted[10,13] that chlamydospore formation by *C. albicans* can be influenced by the variety of corn meal used in the medium. A yellow corn-meal agar employed here has given more consistent results than any other corn-meal medium. This medium is prepared in the following manner:

Preparation of yellow corn-meal agar.—Forty grams of yellow, commercial corn meal (Purity Cornmeal, Purity Flour Mills Ltd., Toronto) are mixed with 500 ml hot distilled water in a large beaker and boiled for 2 minutes over a medium-sized flame. The mixture is allowed to cool and the supernatant is filtered through a thin pad of non-absorbent cotton enveloped in a double layer of gauze. The filtrate is made up to 1000 ml with distilled water, 15 g of agar added, and the whole autoclaved at 15 pounds' pressure for 20 minutes. After cooling to 50 C, the medium is dispensed in 20 ml amounts into sterile petri plates. The pH of the adjusted medium after sterilization is 6.2.

14. Littman, M. L. 1947, A culture medium for the primary isolation of fungi. Science, **106**: 109–111.

In the routine procedure radial cuts approximately 1 to 1.5 cm long and 1 mm deep, starting 1.5 cm from the periphery of the plate, are made with an inoculating needle. Sterile 1.2 ounce cover slips (7/8 inch square) are lowered and pressed gently over the cut area. In agreement with Kligman,[10] the application of a cover slip is recommended since plates can be opened repeatedly, examined microscopically or photographed without contaminating the inoculated areas. The plates are incubated at 22 to 24 C and the inoculated areas are examined microscopically within 24 hours. All plates are kept 7 to 10 days before being discarded.

RESULTS

C. albicans can be identified in 1 to 3 days by the presence of chlamydospores which are thick-walled, spherical, enlarged cells, 6.9 to 17.2 μ in diameter (with or without buds or secondary chlamydospores), borne singly or in bunches on the tips of sausage-shaped or filamentous pseudohyphae (chlamydophores). Other candida species can be identified according to the descriptions outlined by Martin et al.[3]

The rapidity with which candida species isolated from patients can be identified by the above method and the consistency of the results recommends it for routine use in other laboratories.

THE RELATION OF THE TRICARBOXYLIC ACID CYCLE TO BACTERIAL INFECTION

III. COMPARISON OF SURVIVAL TIME OF MICE INFECTED WITH DIFFERENT PATHOGENS AND GIVEN KREBS CYCLE INHIBITORS AND INTERMEDIATES

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The survival time of infected mice is dramatically reduced by certain substances capable of interfering with, or altering, the "normal" functioning of the Krebs cycle (Berry and Mitchell 1953a, b, and c). A relationship of this type was postulated (Berry and Mitchell, 1953d) in an effort to account for the increased susceptibility to typhoid in mice exposed to simulated high altitude, since this greater susceptibility could not be explained by any demonstrable change in cellular defense or humoral defense. This postulate led to the experiments in which the Krebs cycle was modified by injecting, at appropriate intervals, sublethal amounts of one of three inhibitors or one of two intermediates (Berry and Mitchell, 1953b and c). The sodium salts of malonic, fluoroacetic, and arsenious acids were used as inhibitors, and sodium citrate and sodium succinate were used as intermediates. Infections with a standardized suspension of *Salmonella typhimurium* were artificially induced by injecting the bacteria into the abdominal cavity of the mice. Control animals similarly infected were administered a volume of isotonic sodium chloride solution equal to the volume in which the inhibitor or intermediate was carried. Thus, the one variable between experimental and control mice was the presence or absence, in vivo, of inhibitor or intermediate.

Since reductions in survival time of infected mice can result from injections of inhibitors which act on different enzymes (Buffa and Peters, 1950; Peters, Sinclair and Thompson, 1946; Quastel and Whetham, 1925) and which produce in the animal an accumulation of different intermediates (Busch and Potter, 1952a and b; Krebs, Salvin and Johnson, 1938; Lindenbaum, White and Schubert, 1951; Peters and Wilson, 1952; Potter and Busch, 1950), the question arises as to how these substances act.

As a working hypothesis, Berry and Mitchell (1953c) assumed that the five compounds have a common effect within the infected mouse. If there is any validity in this hypothesis, inhibitors and intermediates should vary in their ability to reduce survival time in mice infected with different pathogens. The experiments to be described in this report were designed to test this prediction. For the most part, it is confirmed.

METHODS

Female mice of the CF-1 strain, 6 to 9 weeks old, were used in these experiments. All infections were artificially induced by administering intraperitoneally a standard suspension of bacteria. The compounds to be tested for their effect on survival time were injected by the same route. They were dissolved in sterile saline in such concentration that the desired amount was contained in 0.5 ml. Table 1 gives the pertinent data for each substance.

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TABLE 1.—*The number, time intervals, and dosages of injections of metabolic inhibitors and intermediates.*

Substance	Amount per injection	Total number of injections	Concentration	Time intervals
Saline	½ ml	8	0.9%	1 immediately and hourly for 7
Disodium malonate	½ ml	8	20 mg/½ ml	1 immediately and hourly for 7
Sodium monofluoroacetate	½ ml ±	2	8 mg/kg mouse	1 immediately and 1 after 6 hr
Sodium arsenite	½ ml	3	5.4 mg/kg mouse	1 immediately, 1 after 1 hr, 1 after another 1½ hr
Trisodium citrate	½ ml	8	10 mg/½ ml	1 immediately and hourly for 7
Disodium succinate	½ ml	8	20 mg/½ ml	1 immediately and hourly for 7
Glucose	½ ml	8	10 mg/½ ml	1 immediately and hourly for 7

The bacteria with which the mice were infected were grown at 37 C on brain heart infusion broth (Difco) fortified with 4% blood. Appropriate dilutions were prepared with the same medium minus blood immediately before they were to be used. The organisms employed, the incubation period of the culture, the approximate number of bacteria injected per mouse, as determined by serial dilution plate counts, are summarized in table 2.

For each experiment, groups of 5 mice were established in separate cages. Each small galvanized-iron cage contained wood shavings, Purina dog chow checkers as food, and a bottle of water. For each group that was to be infected and given one of the compounds listed in table 1, there was a corresponding control group which was to receive only the injections of the compound. Thus each inhibitor and intermediate was given to an infected group and to an uninfected group, the latter serving as controls for the toxicity of the compounds. The mice in the 2 groups were selected at random from the total stock of animals obtained in a given shipment from the commercial source so that the individuals weighed within 1 g of a given weight. A single group served as control for the infection, and these animals were injected with a volume of saline equal to that given the experimental mice. It is important to emphasize this point since most of the mice were injected during a period of 7 hours with 4 ml of fluid. The fact that control and experimental mice received a comparable volume of fluid eliminates this as a factor in the final results.

RESULTS

Streptococcus *C203 A3* (*hemolytic*)

The number of mice surviving at the times indicated following their infection with a 1:1 dilution of a 24-hour culture of *Streptococcus* C203 A3 is shown in table 3. Those receiving malonate suffered the first death 4½ to 5 hours after the bacteria had been administered and the last death after 8½ to 10½ hours. By comparison, the first infected control mouse, receiving only injections of sterile saline, died between 8 and 8½ hours. At this time only one of the mice injected with malonate survived and the last control mouse died sometime between 13 and 24 hours.

Mice injected with succinate, citrate, and arsenite survived for periods closely comparable to those for controls. The slight variations that exist are probably to be attributed to individual differences. There is, however, an interesting effect shown by mice given fluoroacetate. All of these animals were alive 5 hours after the first control mouse died and all were still alive when only one of the controls remained. This sug-

TABLE 2.—*Organisms, strains, culture ages, dilutions, and approximate numbers of cells used to infect mice artificially.*

Organism	Strain	Age of culture	Dilution	Approximate number of organisms
Corynebacterium kutscheri	ATCC* 6944	5 hr	Undiluted	
Proteus morganii	ATCC 9237	5 hr	1:100	5 million
Klebsiella pneumoniae	2661	5 hr	1:200	500,000
Micrococcus pyogenes var. *aureus*	ATCC 10,390	5 hr	3 ml in 10	8 million
Diplococcus pneumoniae	SV 1	18 hr	1:100 million	1–10
Streptococcus pyogenes	C 203 A-3	24 hr	1:1	165 million

* American Type Culture Collection.

TABLE 3.—*Mice surviving at indicated time intervals following infection with Streptococcus pyogenes C 203 A-3 and injections of compounds listed. Each substance was administered according to the schedule shown in table 1. There were 5 mice in each group.*

Hours after infection	Malonate		Succinate		Citrate		Arsenite		Fluoroacetate		Infected control
	C	E	C	E	C	E	C	E	C	E	
1 – 4½	5	5	5	5	5	5	5	5	5	5	5
4½– 5	5	4	5	5	5	5	5	5	5	5	5
5 – 6½	5	3	5	5	5	5	5	5	5	5	5
6½– 7	5	2	5	5	5	5	5	5	5	5	5
7 – 7½	5	2	5	4	5	5	5	5	5	5	5
7½– 8	5	1	5	4	5	5	5	5	5	5	5
8 – 8½	5	1	5	4	5	5	5	5	5	5	4
8½–10½	5	0	5	3	5	3	5	5	5	5	4
10½–11	5		5	2	5	3	5	4	5	5	2
11 –12	5		5	2	5	1	5	2	5	5	2
12 –13	5		5	1	5	1	5	2	5	5	1
13 –24	5		5	0	5	0	5	0	5	1	0
24 –120	5		5		5		5		5	1	

C, Control.
E, Experimental.

gests that fluoroacetate protects the mice against infection with this strain of streptococcus, possibly by inhibiting the metabolism of the bacteria more effectively than that of the host.

In a duplicate experiment these results were confirmed. At the same time a group of infected mice was injected at hourly intervals with glucose (cf. table 1), but their survival was not significantly different from that of infected control mice.

Pneumococcus *SV 1* (Diplococcus pneumoniae)

The results obtained when mice were infected with a 1:100,000,000 dilution of an 18-hour culture of *Pneumococcus* SV 1 are recorded in table 4. This number of organisms, administered intraperitoneally, failed to kill any of the control mice, but the groups injected with malonate and with fluoroacetate each lost 2 mice between 5 and 8 hours. One mouse in the group of toxicity controls for fluoroacetate died, while a single animal died during the 3rd day in each of the groups receiving succinate or citrate. Death after such a delay indicates that events taking place while the intermediates were being administered may exert influences not manifested for a number of hours. While survival time in some of these experiments is reduced to less than 12 hours, it is important to recognize the considerable interval that may elapse before the infection is fatal.

TABLE 4.—*Mice surviving at indicated time intervals following infection with* Diplococcus pneumoniae *SV 1 and injections of compounds listed. Each substance was administered according to the schedule shown in table 1. There were 5 mice in each group.*

Hours after infection	Malonate		Succinate		Citrate		Arsenite		Fluoroacetate		Infected control
	C	E	C	E	C	E	C	E	C	E	
1 – 4½	5	5	5	5	5	5	5	5	5	5	5
4½– 5	5	5	5	5	5	5	4	5	5	5	5
5 – 5½	5	4	5	5	5	5	4	5	5	5	5
5½– 7½	5	3	5	5	5	5	2	4	5	4	5
7½– 8	5	3	5	5	5	5	2	4	5	3	5
8 –24	5	3	5	5	5	5	2	4	4	3	5
24 –29	5	3	5	5	5	5	1	4	4	3	5
29 –48	5	3	5	5	5	5	1	3	4	3	5
48 –52½	5	3	5	5	5	4	1	3	4	3	5
52½–54½	5	3	5	4	5	4	1	2	4	3	5
54½–59	5	3	5	4	5	4	1	2	4	2	5
59 –120	5	3	5	4	5	4	1	2	4	2	5

TABLE 5.—*Mice surviving at indicated time intervals following infection with* Proteus morganii *ATCC 9237 and injections of compounds listed. Each substance was administered according to the schedule shown in table 1. There were 5 mice in each group.*

Hours after infection	Malonate		Succinate		Citrate		Arsenite		Fluoroacetate		Infected control
	C	E	C	E	C	E	C	E	C	E	
1 – 5	5	5	5	5	5	5	5	5	5	5	5
5 – 5½	5	4	5	5	5	5	5	5	5	5	5
5½– 7	5	2	5	3	5	5	5	5	5	5	5
7 – 7½	5	1	5	3	5	5	5	5	5	5	5
7½–10	5	0	5	2	5	5	5	5	5	4	5
10 –11	5		5	2	5	5	5	5	5	3	5
11 –12	5		5	2	5	4	5	3	5	3	5
12 –24	5		5	1	5	4	5	1	5	2	5
24 –29	5		5	1	5	4	5	0	4	2	5
29 –120	5		5	1	5	4	5		4	2	5

The survival data of infected mice given arsenite fail to reveal any effect since 4 of 5 toxicity controls died within a period of 29 hours. This is the largest mortality observed with arsenite but a dosage error must have been made.

In a duplicate experiment, no infected control mice died and no toxicity controls for arsenite and for fluoroacetate were lost. None of the infected mice given arsenite died. This confirms the tentative conclusions drawn above. Moreover, 4 of the infected group injected with fluoroacetate died between 26 and 48 hours. These results are even clearer than those shown in table 4.

Glucose was not tested with this organism.

Proteus morganii *ATCC 9237*

Survival of mice infected with a 1:100 dilution of a 24-hour culture of *Proteus morganii* is recorded in table 5. All of the infected control mice survived the entire experimental period, while one of the toxicity controls for fluoroacetate died early the 2nd day. There were no deaths among the other control mice. All infected mice given injections of malonate died between 5 and 10 hours, the minimum survival time for any of the groups. Two members of the group receiving succinate died between 5½ and 7 hours, another between 9 and 10 hours, the 4th between 12 and 24 hours, and one survived. The timing of these deaths indicates a more rapid progress of the infection in the presence of succinate, assuming that the animals died of the infection, than that obtained even with arsenite, in which 4 mice died between 12 and 24 hours and the last at 29 hours. In experiments of this type, great significance cannot be attached to death of a single animal because of potentially large individual differences.

Fluoroacetate injections in infected mice resulted in the death of 3 of the group during the 1st day. The timing of these deaths was roughly comparable to those in the group receiving succinate. The mice given injections of citrate, on the other hand, the compound known to accumulate in vivo in fluoroacetate poisoning (Buffa and Peters, 1950), had a single death between 11 and 12 hours, and the remaining 4 survived.

These results were confirmed in a duplicate experiment. It is, therefore, possible to conclude that malonate is most effective in reducing survival time of mice infected with *Proteus morganii*. Glucose injections were without effect in the separate experiment in which it was tested.

Micrococcus pyogenes *var.* aureus *ATCC 10390*

The results obtained with mice infected with a 3:13 dilution of a 5-hour culture of *Micrococcus pyogenes* are recorded in table 6. Malonate is markedly

TABLE 6.—*Mice surviving at indicated time intervals following infection with* Micrococcus pyogenes *var.* aureus (Staphylococcus aureus) *and injections of compounds listed. Each substance was administered according to the schedule shown in table 1. There were 5 mice in each group.*

Hours after infection	Malonate		Succinate		Citrate		Arsenite		Fluoroacetate		Infected control
	C	E	C	E	C	E	C	E	C	E	
1 – 5	5	5	5	5	5	5	5	5	5	5	5
5 – 6	5	2	5	5	5	5	5	5	5	5	5
6 – 6½	5	0	5	5	5	5	5	5	5	5	5
6½– 7½	5		5	5	5	4	5	5	5	5	5
7½– 8½	5		5	5	5	4	5	5	5	4	5
8½–10	5		5	5	5	3	5	2	5	2	5
10 –12	5		5	5	5	2	5	1	5	0	5
12 –24	5		5	5	5	1	5	0	4		5
24 –96	5		5	5	5	0	5		4		5
96 –120	5		5	5	5		5		4		5

effective in causing the death within 6½ hours of all 5 infected mice receiving it. None of the infected control mice died at any stage of the experiment. Succinate exerted no influence on this infection, while citrate, arsenite, and fluoroacetate were all active in reducing survival time. The two inhibitors act more rapidly than citrate, but this is due primarily to the survival of a single animal. Arsenite and fluoroacetate give almost identical mortality rates. The death of a fluoroacetate toxicity control mouse came after the deaths of all mice in the infected group so it does not alter the validity of the results. Glucose was completely without effect.

The results described above were confirmed in a duplicate experiment. Therefore it is safe to conclude that malonate exerts the greatest influence on the mortality of mice infected with *Micrococcus pyogenes*. Fluoroacetate, citrate, and arsenite also "enhance" the infection but to a lesser degree than malonate.

Corynebacterium kutscheri *ATCC 6944*

The results obtained when mice were infected with an undiluted 5-hour culture of *Corynebacterium kutscheri* are recorded in table 7. Arsenite was the most effective of the compounds tested. Three of the experimental mice injected with arsenite were dead between 28 and 48 hours while, at this time, all of the infected control mice survived. Subsequently, between 54 and 60 hours, all arsenite-poisoned mice were dead, while 3 control mice remained. Citrate was also very effective, showing a pattern closely paralleling that of arsenite. Malonate and succinate produced almost identical mortalities until the end of the 3rd day, when the latter became more effective. Fluoroacetate was not able to alter the mortality rate to any

TABLE 7.—*Mice surviving at indicated time intervals following infection with* Corynebacterium kutscheri *ATCC 6944 and injections of compounds listed. Each substance was administered according to the schedule shown in table 1. There were 5 mice in each group.*

Hours after infection	Malonate		Succinate		Citrate		Arsenite		Fluoroacetate		Infected control
	C	E	C	E	C	E	C	E	C	E	
1 –10	5	5	5	5	5	5	5	5	5	5	5
10 –12	5	5	5	5	5	5	5	5	5	3	5
12 –24	5	5	5	5	5	5	5	5	3	2	5
24–28	5	5	5	5	5	5	5	4	3	2	5
28–48	5	3	5	4	5	1	5	2	3	2	5
48–54	5	2	5	2	5	1	5	1	3	2	4
54–60	5	2	5	2	5	1	5	0	3	2	3
60–72	5	2	5	0	5	0	5		3	1	1
72–96	5	1	5		5		5		3	0	1
96–120	5	1	5		5		5		3		1

TABLE 8.—*Mice surviving at indicated time intervals following infection with* Klebsiella pneumoniae *2661 and injections of compounds listed. Each substance was administered according to the schedule shown in table 1. There were 5 mice in each group.*

Hours after infection	Malonate		Succinate		Citrate		Arsenite		Fluoroacetate		Infected control
	C	E	C	E	C	E	C	E	C	E	
1 – 4½	5	5	5	5	5	5	5	5	5	5	5
4½– 5½	5	4	5	5	5	5	4	5	5	5	5
5½– 8	5	4	5	5	5	5	4	5	5	4	5
8 –10½	5	3	5	5	5	5	4	5	5	4	5
10½–12	5	2	5	5	5	5	4	5	5	4	5
12 –24	5	1	5	1	5	5	4	3	5	0	4
24 –26	5	1	5	1	5	4	4	2	5		4
26 –48	5	1	5	1	5	3	4	2	5		4
48 –120	5	1	5	1	5	3	4	2	5		4

significant degree so the loss of 2 toxicity controls is not important for the results. The effect of glucose was not tested with this organism and confirming tests were not undertaken.

Klebsiella pneumoniae *2661*

The results of infections using a 1:200 dilution of a 5-hour culture of *Klebsiella pneumoniae* are recorded in table 8. Malonate and fluoroacetate are the most effective in reducing survival time, causing 4 and 5 deaths, respectively, in the first 24 hours while only one of the infected control mice died. One mouse receiving malonate and 4 controls survived. Succinate was very effective between 12 and 24 hours, during which time 4 mice died. Citrate and arsenite were less effective than the compounds already mentioned although both showed some influence. The loss of one toxicity control in the arsenite group does not alter the validity of this conclusion. Glucose was without effect.

These results were successfully confirmed. It can be concluded that malonate causes the most immediate effect although fluoroacetate and succinate are active a few hours later.

DISCUSSION

The results of the experiments described in this report show that the reduction in survival time of infected mice given injections of malonate, fluoroacetate, arsenite, citrate, or succinate depends upon the particular bacterial pathogen with which the animals are infected. In some cases the reduction is so obvious that statistical analysis seems unnecessary. For example, when all of 5 mice in one group are dead and all of another survive, the probability that this occurs by chance alone is less than 0.01, as determined by the chi-square test. In other cases, like the malonate and control groups in table 4, the differences cannot be considered significant until additional work with larger numbers of mice has been completed, even though duplicate experiments yield comparable results. At this stage of our understanding of these phenomena, it is much more important to know that malonate (and each of the other compounds) is not equally effective in all infections than it is to know with certainty whether a substance does or does not result in a slight but significant increase in mortality with a given pathogen. The over-all pattern of survival of the groups of mice infected with the different organisms is the important picture these data convey. Each pattern is unique. The causative organism in an infection could be identified readily by data like those in tables 3 to 8, alone.

The findings now at hand make this apparent. All 5 compounds reduce survival time in mice infected with *S.*

typhimurium (Berry and Mitchell, 1953b and c) or with *Klebsiella pneumoniae* (table 8), but malonate is the most potent with the former and fluoroacetate with the latter. With *Proteus morganii*, all compounds but citrate are effective (table 5), while with *Micrococcus pyogenes* all but succinate (table 6) are active. Citrate and arsenite reduce survival time in animals infected with *Corynebacterium kutscheri* (table 7) and malonate, alone, promotes infections with streptococci (table 3). Mice infected with pneumococci (table 4) are not influenced by any one compound as noticeably as some of the above, even though there are some deaths in groups with modified Krebs cycle and none in the infected controls.

It is for these reasons, therefore, that the underlying mechanism of action of these compounds in reducing survival time seems to depend upon a specific link between the biochemical effect of the compound injected and the biochemistry of host-parasite interaction. If one assumed, for example, that malonate, which is effective with 6 of the 7 pathogens investigated, inhibits the cellular defense of the mouse, then why would it be ineffective with even one pathogen?

Attention is directed also to the fact that control mice infected with pneumococci, *Proteus morganii*, or *Micrococcus pyogenes* suffered no deaths during the first week postinfection, the arbitrary limit of these experiments. Animals similarly infected and injected with certain of the compounds died within a few hours. Since a sublethal infection becomes lethal in the presence of sublethal amounts of inhibitor or intermediate of the Krebs cycle, some kind of summation of action must be postulated. The results of experiments, now being prepared for publication, will help to clarify these relationships.

SUMMARY

The effect of injections of malonate, fluoroacetate, arsenite, citrate, and succinate on the survival time of mice infected with each of the following organisms was determined: *Streptococcus* C203 A3 hemolytic, *Diplococcus pneumoniae*, *Proteus morganii*, *Micrococcus pyogenes* var. *aureus*, *Corynebacterium kutscheri*, and *Klebsiella pneumoniae*. In mice infected with streptococci, malonate alone was able to reduce survival time, while both malonate and fluoroacetate were moderately active in pneumococci infections. All compounds but citrate enhanced infections with proteus, and all but succinate were effective in mice infected with *Micrococcus pyogenes*. Arsenite and citrate each combined with corynebacteria to reduce survival time, while all were active in infections with klebsiella. Glucose was completely inactive in the four infections in which it was tested. A specificity of action seems to exist, therefore, between each of the pathogens and certain of the five compounds listed, and this action may, in some cases, convert an otherwise sublethal infection into one that is quickly lethal.

REFERENCES

Berry, L. J. and Mitchell, R. B. 1953a, The effect of malonate on *Salmonella typhimurium* infection in mice. Science, **118**: 140–141.

———. 1953b, The relation of the tricarboxylic acid cycle to bacterial infection. I. The effect of malonate on *Salmonella typhimurium* infections in mice. J. Infect. Dis. **93**: 75–82.

———. 1953c, The relation of the tricarboxylic acid cycle to bacterial infection. II. The effect of fluoroacetate, arsenite, citrate, and succinate on *Salmonella typhimurium* infections in mice. J. Infect. Dis. **93**: 83–92.

———. 1953d, Influence of simulated altitude on resistance-susceptibility to *Salmonella typhimurium* infection in mice. Texas Reports Biol. & Med. **11**: 379–401.

Buffa, P. and Peters, R. A. 1950, The in vivo formation of citrate induced by fluoroacetate and its significance. J. Physiol. **110**: 488–500.

Busch, H. and Potter, V. R. 1952a, Studies on tissue metabolism by means of in vivo metabolic blocking technics. I. A survey of changes induced by malonate in tissues of tumor-bearing rats. Cancer Res. **12**: 660–665.

———. 1952b, Succinate accumulation in vivo following injections of malonate. J. Biol. Chem. **198**: 71–77.

Krebs, H. A., Salvin, E. and Johnson, W. A. 1938, The formation of citric and alpha-ketoglutaric acids in the mammalian body. Biochem. J. **32**: 113–117.

Lindenbaum, A., White, M. R. and Schubert, J. 1951, Citrate formation in vivo induced by non-lethal doses of fluoroacetate. J. Biol. Chem. **190**: 585–593.

Peters, R. A., Sinclair, H. M. and Thompson, R. H. S. 1946, An analysis of the inhibition of pyruvate by arsenicals in relation to the enzyme theory of vesication. Biochem. J. **40**: 516–524.

Peters, R. A. and Wilson, T. H. 1952, A further study of the inhibition of aconitase by inhibitor fraction isolated from tissues poisoned with fluoroacetate. Biochim. et Biophys. Acta. **9**: 310–315.

Potter, V. R. and Busch, H. 1950, Citric acid content of normal and tumor tissues in vivo following injections of fluoroacetate. Cancer Res. **10**: 353–356.

Quastel, J. H. and Whetham, M. D. 1925, Dehydrogenations produced by resting bacteria. Biochem. J. **19**: 520–531.

THE RELATION OF THE TRICARBOXYLIC ACID CYCLE TO BACTERIAL INFECTION

IV. THE EFFECT OF THREE METABOLIC INHIBITORS AND *SALMONELLA TYPHIMURIUM* ON THE CITRIC ACID CONTENT OF MOUSE TISSUES

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Experiments to determine the effect of changes in metabolism induced by means of specific inhibitors upon the response to infection were first reported by Ackermann (1951). He administered to mice sublethal doses of sodium fluoroacetate, which blocks the tricarboxylic acid cycle and causes citric acid to accumulate in the lungs. Under these conditions, the propagation of type A influenza virus, artificially introduced into the mice by nasal insufflation, was markedly inhibited.

More recently experiments have been conducted (Berry and Mitchell, 1953a and b) which show that mice are more susceptible to bacterial infection when the citric acid cycle is partially blocked. The administration of three inhibitors of the cycle, sodium malonate, sodium arsenite, and sodium fluoroacetate, in sublethal doses, results in a reduction of survival time in mice infected with *Salmonella typhimurium*. Such intermediates of the Krebs cycle as succinate and citrate also produce this effect. When the same compounds were tested in mice infected with one of several different pathogens, Berry, Merritt and Mitchell (1954) found that at least one inhibitor or intermediate was able to reduce the survival time of the infected animals. These experiments demonstrate that a relationship exists between the metabolic processes of an animal and its resistance to bacterial infection.

In this paper an attempt has been made to determine the effect of three metabolic inhibitors, a pathogenic and a nonpathogenic organism, and a combination of pathogen plus inhibitor on the tricarboxylic acid cycle of the albino mouse. For this purpose citric acid was assayed because of its strategic position as the first compound in the cycle (Peters, Wakelin and Buffa, 1953) and because a good colorimetric method is available for micro-amounts of the compound. The metabolic poisons used in this study were sodium malonate, sodium arsenite, and sodium fluoroacetate.

Sodium malonate blocks the conversion of succinic acid to fumaric acid by the competitive inhibition of succinic dehydrogenase (Baldwin, 1952). Arsenite inhibits the irreversible oxidative decarboxylation of α-ketoglutaric acid (Krebs, 1933) and blocks pyruvic acid oxidase and dehydrogenase (Peters, Sinclair and Thompson, 1946). Sodium fluoroacetate has no action on the "condensing enzyme" but it combines with oxaloacetate to form fluorocitrate (Peters, Wakelin and Buffa, 1953). This compound then exerts a powerful, competitive inhibition of aconitase, thereby checking further metabolism of citrate.

The data to be reported below show that citric acid accumulates in mouse

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tissues following the injection of each of the three inhibitors and of heat-killed *S. typhimurium* antigen.

METHODS

The citric acid content of tissues was determined by the pentabromoacetone method as modified by Natelson, Lugovoy and Pincus (1947). A Beckman DU spectrophotometer was used for all readings.

The six mouse tissues chosen for assay were blood, spleen, kidney, heart, liver, and duodenum. The available literature on the inhibitors (cf. Berry and Mitchell, 1953a and b) used in these investigations indicates that spleen, kidney, and heart best show increases in citric acid due to these inhibitors. Liver, blood, and duodenum were included because *S. typhimurium* is an intestinal parasite which ultimately invades the blood stream and frequently produces liver abscesses. At first, the citric acid was determined for individual samples of each of the six tissues. Since a wide range of values was obtained, as shown in table 1, it was decided that average values could be determined more quickly by pooling the individual tissues of five mice for a single determination.

Blood obtained by decapitation and collected in a paraffined watch glass was measured in a tuberculin syringe. The volumes ranged from 0.25 to 0.5 ml, and when analyses were made on single samples, water was added to make 1.0 ml. Into this, 5 ml of 10% trichloroacetic acid were blown rapidly in order to precipitate the proteins as fine particles. The mixture was shaken mechanically on a Boerner machine, centrifuged, and a 5 ml aliquot of the supernatant liquid used for the assay. The organs were weighed on a torsion balance and transferred to a homogenizing tube surrounded by cracked ice and containing 1 ml of cold isotonic potassium chloride solution (0.9%). When the tissues had been collected, they were homogenized and an equal volume of 20% trichloroacetic acid was added. This mixture was stirred, centrifuged, and 1 ml of the supernatant removed for the citric acid assay.

In the preparation of pooled samples, 0.25 to

TABLE 1.—*The citric acid content of 6 mouse tissues under normal and experimental conditions was determined photometrically. The values are expressed as μg of citric acid per ml of blood or per g wet weight of tissue. Average values are italicized for both individual and pooled samples.*

	Experimental condition	Blood		Spleen		Kidney		Heart		Duodenum		Liver	
	Normal values (individual)	35		92		60		81		100		65	
		40		77		97		124		161		173	
		44		97		72		101		149		129	
I		46	*42*	62	*94*	56	*76*	130	*111*	119	*132*	70	*109*
		48		95		52		120					
		37		139		102							
		42				90							
	Sodium malonate (individual)	29		267		216		215					
		35		142	*205*	377	*297*	198	*166*				
		22	*32*					85					
II		29											
		23											
		53											
	(pooled)	38	*43*	330	*245*	160	*225*	100	*120*	110	*115*	200	*170*
		48		160		290		140		120		140	
	Sodium arsenite (individual)	41	*39*	152	*203*	164	*297*	178	*207*				
III		37		253		429		236					
	(pooled)	56	*56*	190	*185*	350	*425*	130	*150*	230	*230*	260	*280*
		55		180		500		170		230		300	
	Sodium fluoroacetate (individual)	94	*96*	1055	*1005*	797	*938*	743	*631*				
IV		97		956		1079		518					
	(pooled)	77	*77*	800	*795*	1420	*1560*	400	*660*	400	*570*	320	*740*
		77		790		1700		920		740		1160	
	S. typhimurium (individual)	47		147		151		171		320		134	
		66	*54*	111	*160*	90	*125*	442	*270*	99	*235*	227	*289*
		49		209		91		198		286		505	
V				171		169							
	(pooled)	47		490		170		100		200		240	
		59	*50*	170	*273*	230	*190*	310	*187*	180	*170*	470	*443*
		43		160		170		140		130		620	
	S. typhimurium and malonate (pooled)	56	*70*	190		190		510		440		120	
VI		83		180	*173*	170	*173*	330	*407*	590	*543*	310	*240*
				150		160		380		600		290	
VII	*A. aerogenes* (pooled)	30		100		90		90		90		240	

0.5 ml of blood was collected from each animal and put into one flask. A volume of water equal to the volume of blood was added and 10 times this amount of 10% trichloroacetic acid was blown into the flask. Following shaking and centrifugation, a 5 ml aliquot was used for the assay. Each of the other 5 tissues was weighed and transferred to a homogenizing tube for that particular tissue, which contained 1 ml of cold isotonic potassium chloride solution for each 100 mg of organ. The tubes were kept in cracked ice throughout this procedure. After homogenization, an equal volume of 20% trichloroacetic acid was added. This was stirred, centrifuged, and 1 ml of supernatant used for the assay.

The inhibitors in these experiments were injected intraperitoneally in the following sublethal dosages: sodium malonate, 20 mg; sodium arsenite, 0.125 mg; and sodium fluoroacetate, 8 mg per kg body weight. The results of previous experiments (Berry and Mitchell, 1953a and b) show these amounts to be effective in reducing the survival time of mice infected with *S. typhimurium*. The inhibitors were dissolved in sterile physiological saline (0.9% sodium chloride) so that the desired quantity was contained in 0.5 ml. The mice were sacrificed 1 hour after injection.

The suspensions of *S. typhimurium* and of the nonpathogen, *Aerobacter aerogenes*, were prepared by inoculating 10 ml of brain heart infusion broth (Difco) and incubating for 15 to 19 hours at 37° C. The number of bacteria contained in these cultures was about 1.5 billion per ml, as determined by serial dilution counts. The organisms were killed by heating the cultures in a water bath at 60° to 70° C for 1 hour. Sterility was proven by the absence of growth in subcultures. They were stored in the refrigerator until used. One ml of the suspension was injected intraperitoneally into mice about 15 hours before assays were carried out on the tissues.

An experiment was conducted to show whether the combined effect of heat-killed *S. typhimurium* and sodium malonate reduces survival time by an amount comparable to that caused by living bacteria and malonate. One ml of the heat-killed suspension was administered to each of 20 mice. After 15 hours, half of the mice were given hourly injections of 20 mg malonate contained in 0.5 ml of saline and the remaining half were given the same number of injections of saline. A third group of 5 mice was given hourly injections of malonate.

Young adult female mice of the CF-1 strain were used. The body weights of these mice varied from 20 to 30 g. Although no apparent correlation was found between the weight of the mouse and the citric acid content of its organs in the individual determinations, in the experiments using pooled tissues of 5 mice, animals were selected with body weights differing by only 1 g.

RESULTS

Values for the normal citric acid content of the tissues are reported in the first row (horizontal) of table 1. They are expressed as μg per ml of blood or per g wet weight of tissue. The range of values for all tissues, except blood, indicates that there is considerable individual variation. The maximum value may be more than double the minimum value.

In the second row, the effect after 1 hour of an injection of 20 mg malonate on the citric acid content of tissues is reported. The spleen (2.6 times normal), the kidney (3.0 times normal), and the liver (1.6 times normal) show definite increases that are statistically significant, with a probability less than 0.02 as determined by the *t* test. The heart, duodenum, and blood were within the normal range. The individual determinations made on blood, spleen, kidney, and heart correspond well with the pooled determinations, except for the heart, which showed a slight increase (1.5 times normal) and the kidney, which accumulated citric acid to a concentration 3.9 times the normal value for that tissue.

The third row of table 1 contains the values for citric acid concentration in tissues 1 hour after 0.125 mg of arsenite had been injected. The kidney showed the largest increase (5.6 times normal). The liver had a citric acid content 2.6 times normal, while the concentration in the spleen doubled. In the duodenum it was 1.7 times normal, and in the heart it was 1.4 times normal. The concentration of citric acid in the pooled samples of blood was higher than normal (56 μg per ml vs. 42 μg per ml). The two indi-

vidual samples showed no elevation, but when these are combined with the pooled samples and tested for significance by the *t* test, a probability of less than 0.01 is obtained. The same significance was also found for the other samples. The individual determinations on spleen and heart were in close agreement with the pooled values, but the two individual kidney determinations were lower (3.9 times normal).

The citric acid concentration of tissues 1 hour after the injection of 8 mg per kg body weight of fluoroacetate is recorded in row 4 of table 1. The effect of this inhibitor was much more pronounced than that of either of the two preceding compounds, and the assay values are unequivocally greater than those for control mice. In the kidney there was an accumulation of citric acid 21 times the normal value. In all the other tissues, concentrations far above the normal range were found. The spleen was 8.5 times normal, the heart was 6.0, duodenum 4.3, and the liver 6.8 above normal. Even the blood in which no increase was noted with malonate and a slight, but significant one, with arsenite, now showed a definite increase to 77 μg per ml. in the pooled samples and 96 μg per ml in the 2 individual samples. The averages of the separate determinations for spleen and heart agree with the pooled samples, while that for kidney was only 12.3 times normal.

In row 5 of table 1, the citric acid concentration in tissues 15 hours after the intraperitoneal injection of 1 ml of the heat-killed suspension of *S. typhimurium* is shown. Increases were noted in all the tissues to the following extent: spleen 2.9 times normal, kidney, 2.5, heart, 1.7, duodenum, 1.3, and liver, 4.1. The individual values obtained with spleen, kidney, and liver were somewhat lower than these but they were greater than normal values. The citric acid concentration of the blood was 54 μg per ml in individual samples, and 50 μg per ml in pooled samples. All of these values are significantly greater than those for normal mice (probability less than 0.01 by the *t* test). Blood citric acid is not subject to the large changes noted in other tissues. This may be due to the fact that the change is extrinsic to blood and intrinsic to the tissues.

Row 6 of table 1 shows the effect of 1 ml of a suspension of heat-killed *S. typhimurium* plus 20 mg of malonate on the citric acid content of the tissues. The bacteria were injected 15 hours prior to the injection of malonate, and the mice were sacrificed 1 hour after malonate. In heart and duodenum, the concentration of citric acid was greater than with either of these substances alone, while the other tissues showed increases more like those obtained with either malonate or bacteria alone. The spleen was 1.8 times normal, kidney, 2.3, liver, 2.2, heart, 3.7, and duodenum, 4.1. Blood citric acid was almost as high as it was with fluoroacetate. All values were significant to less than 0.01 as determined by the *t* test.

The effects of injecting 1 ml of a heat-killed suspension of *Aerobacter aerogenes* on the citric acid concentration of mouse tissues are shown in row 7 of table 1. The values are within the normal range. This particular nonpathogenic organism is unable, therefore, to alter metabolism insofar as this is revealed by the citric acid assay.

The data in table 2 were obtained with 20 mice injected intraperitoneally with 1 ml of a suspension of heat-killed *S. typhimurium*. After 15 hours, 19 survived. Ten were then given hourly injections of malonate and 9 were given hourly injections of saline. Five normal mice received the malonate injections

and served, therefore, as toxicity controls. All animals were inoculated with the same volume of fluid. As table 2 shows, the survival of mice given the killed bacteria was dramatically reduced by the inhibitor. One mouse died after the 1st injection, 2 after the 2nd, 4 after the 3rd, and 2 after the 4th. The last mouse succumbed only after a 5th and a 6th injection were given. All of the mice that received the endotoxin plus the 6 injections of saline, as well as the malonate controls, survived at this time. The control mouse that died 8 hours after the last saline injection apparently died from internal hemorrhage, as revealed by autopsy. However, the mouse that died 20 hours after the last saline ininjection seems to have succumbed to the bacterial toxin itself.

These results are not contradictory to those reported in a previous paper (Berry and Mitchell 1953a) since only 250,000 heat-killed bacteria were introduced into the mice in the earlier experiments and about 1.5 billion in the present one.

DISCUSSION

The increase in concentration of citric acid in tissues following the administration of malonate, arsenite, or fluoroacetate demonstrates the ability of these compounds to block the citric acid cycle. Even though there is some specific knowledge concerning the enzymes each of these inhibitors attacks (see the introduction), it is still not possible to explain the precise sequence that leads in vivo to the accumulation of citric acid. We can more nearly approximate this with fluoroacetate than with the other compounds because of the brilliant work of Peters and his collaborators (Peters, 1952), but remaining unanswered is the reason for the variation in sensitivity of different organs. Also unanswered is the mechanism by means of which these compounds reduce the survival time of mice infected with *S. typhimurium* (Berry and Mitchell, 1953a and b). The data of table 1 combined with the results of the earlier studies make it apparent that no direct link exists between the effectiveness of a substance in blocking the tricarboxylic acid cycle, as measured by the increased concentration of citric acid, and its ability to reduce survival time. Malonate, for example, is least active in the former respect and most potent in the latter. Tests for other substances of metabolic importance might yield valuable information in this area.

The accumulation of citric acid in the organs of mice inoculated with heat-killed cells of *S. typhimurium* reveals a biochemical disturbance not previously reported. It might have been anticipated, however, from the work of Kun and Miller (1948), who found that the endotoxins of meningococcus and *Salmonella aertrycke* markedly inhibit succinic dehydrogenase in the muscle and liver of rabbits. This also offers an explanation of why the concentration of citric acid in certain organs of mice given injections of both *S. typhimurium* and malonate is larger than with either alone. Each blocks the same enzyme. Why organs should differ so much in their response to both inhibitions cannot be answered at this time.

Endotoxins from gram-negative bacteria are known to produce a variety of biochemical disturbances in experimental animals. One of the earliest reported in the literature was that occurring in blood sugar. There is an initial hyperglycemia followed, in some cases, but not all, by hypoglycemia (Menten and Manning, 1924; Zeckwer and Goodell, 1925; Delafield, 1932; Boivin and Mesrobeanu, 1934; Oddy and Evans, 1940; Koch and Olitzki, 1946; Kun and Miller, 1948). Glycogen

TABLE 2.—*The survival of mice injected with heat-killed* S. typhimurium *followed after 15 hours with either injections of malonate or of isotonic saline.*

Time in hours	Infected control group		Infected experimental group		Poison control group	
	Injected with	Number alive	Injected with	Number alive	Injected with	Number alive
0	*S. typhimurium*	10	*S. typhimurium*	10		
15.0	Saline	9	Malonate	10	Malonate	5
15.5		9		9		5
16.0	Saline	9	Malonate	9	Malonate	5
16.5		9		7		5
17.0	Saline	9	Malonate	7	Malonate	5
17.5		9		5		5
18.0	Saline	9	Malonate	3	Malonate	5
18.5		9		2		5
19.0	Saline	9	Malonate	1	Malonate	5
20.0	Saline	9	Malonate	1	Malonate	5
20.5		9		0		5
28.0		8				5
40.5		7				5
120.0		7				

reserves are depleted by the time the hypoglycemia is established (Kun and Miller, 1948; Kun, 1948a; and Kun and Abood, 1949). There is also an impairment in pyruvate oxidation (Kun and Abood, 1948), an increase in the lactic acid content of blood and tissues, and an elevation in organic phosphate of blood (Kun and Miller, 1948). Kun (1948b) also found that meningococcal endotoxin results in a 28 to 36% inhibition of glucose utilization by rat muscle extracts, which indicates a reduction in hexokinase activity that can be overcome by the addition of Zn-free insulin.

While these effects of bacterial endotoxins are fragmentary and fail to fit into any kind of picture of host-parasite interaction, they represent a promising beginning for a valuable approach to the problem.

SUMMARY

Citric acid was assayed colorimetrically in six organs and tissues of mice. The acid accumulates in liver, kidney, spleen, heart, duodenum, and blood following the intraperitoneal injection of three metabolic inhibitors (sodium malonate, sodium arsenite, and sodium fluoroacetate) in dosages known from previous work to be capable of reducing the survival time in mice infected with *Salmonella typhimurium*. The particular tissue affected and the degree of accumulation varies with the inhibitor. Statistically significant increases in the citric acid content of the organs of mice were also observed when a suspension of heat-killed *S. typhimurium* was introduced into the mice. Killed cells of the nonpathogen *Aerobacter aerogenes* produced no change in the citric acid content of the same tissues. A summation of citric acid accumulation, one due to a metabolic inhibitor and the other to the action of a pathogenic organism, was demonstrated in at least two of the tissues. This type of summation of metabolic blockage may help to explain why mice injected with heat-killed *S. typhimurium* and then given 6 hourly injections of 20 mg of sodium malonate, starting 15 hours after the bacteria were injected, have a reduced survival time as compared with mice given only the antigen and an equal number of injections of sterile saline.

REFERENCES

Ackermann, W. W. 1951, The relation of the Krebs cycle to viral synthesis. II. The effect of sodium fluoroacetate on the propagation of influenza virus in mice. J. Exper. Med. **93**: 635–642.

Baldwin. E. 1952, Dynamic aspects of biochemistry, ed. 2, Cambridge University Press.

Berry, L. J. and Mitchell, R. B. 1953a, The relation of the tricarboxylic acid cycle to bacterial infection. I. The effect of malonate on *Sal-*

monella typhimurium infections in mice. J. Infect. Dis. **93**: 75–82.

Berry, L. J. and Mitchell, R. B. 1953b, The relation of the tricarboxylic acid cycle to bacterial infection. II. The effect of fluoroacetate, arsenite, citrate, and succinate on *Salmonella typhimurium* infections in mice. J. Infect. Dis. **93**: 83–92.

Berry, L. J., Merritt, P. and Mitchell, R. B. 1954, The relation between the tricarboxylic acid cycle to bacterial infection. III. Comparison of survival time of mice infected with different pathogens and given Krebs cycle inhibitors and intermediates. J. Infect. Dis. **94**: 144–151.

Boivin, A. and Mesrobeanu, L. 1934, Sur l'action hyperglycemiante du complexe toxique, specifique et antigenique isole a partir du bacille d'Aertrycke. Compt. rend. Soc. de biol. **117**: 273–275.

Delafield, M. E. 1932, A comparison of the changes in the blood sugar and blood phosphorus in rabbits following the injection of suspensions of different dead bacteria. J. Path. & Bact. **35**: 53–68.

Koch, P. K. and Olitzki, L. 1946, The action of dysentery toxins on different laboratory animals. Exper. Med. & Surg. **4**: 54–68.

Krebs, H. A. 1933, Untersuchungen über die Stoffwechsel die Aminosäuren im Tierkörper. Ztschr. f. physiol. Chem. **217**: 191–227.

Kun, E. 1948a, Effect of bacterial endotoxins on glycogen synthesis. Proc. Soc. Exper. Biol. & Med. **68**: 496–498.

———. 1948b. Inhibition of the phosphorylation of glucose by meningococcal endotoxin. J. Biol. Chem. **174**: 761–762.

Kun, E. and Abood, L. G. 1949, Mechanism of inhibition of glycogen synthesis by endotoxins of *Salmonella aertrycke* and type I meningococcus. Proc. Soc. Exper. Biol. & Med. **71**: 362.

Kun, E. and Miller, C. P. 1948, Effect of bacterial endotoxins on carbohydrate metabolism of rabbits. Proc. Soc. Exper. Biol. & Med. **67**: 221–225.

Menten, M. L. and Manning, H. M. 1924, Blood sugar studies on rabbits infected with organisms of the enteritidis-paratyphoid B group. J. Med. Res. **44**: 675–676.

Natelson, S., Lugovoy, J. K. and Pincus, J. B. 1947, Determination of micro quantities of citric acid in biological fluids. J. Biol. Chem. **170**: 597–606.

Oddy, J. G. and Evans, D. G. 1940, The effects produced by toxic and non-toxic extracts of *H. pertussis* and *Br. bronchiseptica* on the blood sugar of rabbits. J. Path. & Bact. **50**: 11–16.

Peters, R. A. 1952, Lethal synthesis. Proc. Roy. Soc., London, s.B. **139**: 143–170.

Peters, R. A., Sinclair, H. M. and Thompson, R. H. S. 1946, An analysis of the inhibition of pyruvate by arsenicals in relation to the enzyme theory of vesication. Biochem. J. **40**: 516–524.

Peters, R. A., Wakelin, R. W. and Buffa, P. 1953, Biochemistry of fluoroacetate poisoning. Proc. Roy. Soc., London, s.B. **140**: 97–507.

Zeckwer, I. T. and Goodell, H. 1925, Blood sugar studies. I. Rapid alterations in the blood sugar level of rabbits as a result of intravenous injections of killed bacteria of various types. J. Exper. Med. **42**: 43–56.

THE EFFECT OF SPLENECTOMY OR PHENYLHYDRAZINE ON INFECTIONS WITH *PLASMODIUM BERGHEI* IN THE WHITE MOUSE

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A previous investigation[1] on the effect of X irradiation on infections with *Plasmodium berghei* in the white mouse led to the observation that X-rayed animals exhibited a lower parasitemia than control animals. This was believed due to radiation damage of erythropoietic tissue and to the subsequent inability of the host to supply suitable substrate, or immature red cells, for the maintenance of the parasite. Because of this it was tentatively assumed that other changes imposed on the erythropoietic tissue of the host animals should be reflected in the degree of parasitemia. To test this hypothesis, erythroid tissue was either reduced in amount by surgical procedures or stimulated to greater activity by chemical means. In view of the important role played by the spleen in supplying erythrocytes to infected animals,[2] this was the organ chosen for removal. By this means not only was erythropoietic tissue eliminated but the erythropoietic potential of the host was decreased. To induce hyperplasia of the erythroid elements of both the spleen and bone marrow, several other groups of mice were subjected to either phenylhydrazine hydrochloride or acetyl phenylhydrazine. The administration of these drugs resulted in an anemia and its attendant reticulocytosis. In accordance with the assumption mentioned earlier, the splenectomized animals were unable to support as intense an infection as were the controls, while the drug-treated animals with hyperplastic erythroid tissue showed a higher parasitemia than did control mice.

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1. Singer, I. 1953, J. Infect. Dis. **92**: 97–104.
2. Singer, I. 1954, J. Infect. Dis. In press.

MATERIALS AND METHODS

The KBG 173 strain of *P. berghei* was obtained from the National Institutes of Health on May 7, 1951 and has been passed biweekly in this laboratory in white mice by the intraperitoneal route. Infected animals have been kept in air-conditioned quarters, at about 72 F, with alternating periods of 12 hours of light and 12 hours of darkness.

Five- to six-week-old CF1 female mice obtained from Carworth Farms were used in all experiments. To initiate infection 12,500,000 parasites were given intravenously. The desired inoculum was obtained by dilution of the donors' blood after red cell and parasite counts had been made.

Smears for parasite counts, smears for reticulocyte counts, and red cell counts were made daily of blood taken from the tail veins of the animals. Smears for reticulocytes were made over a dried film of 0.3% (in 95% ethyl alcohol) brilliant cresyl blue and were placed in a moist chamber for 5 to 10 minutes. On removal from the moist chamber they were rapidly dried and examined later on the same day. Reticulocytes are expressed as the percentage of red cells. The smears for parasite counts were dried, fixed in methyl alcohol and stained with Giemsa. Parasite counts were made according to the method used by Taliaferro and

Taliaferro[3] and are expressed as the number of parasites per 10,000 red blood cells.

Two groups of 10 animals each were prepared for surgery 2 days before infection. One group was marked for splenectomy and the other for nephrectomy. The nephrectomized animals were to serve as controls to be certain that surgical trauma and the absence of a mass of tissue not concerned with erythropoiesis did not affect parasitemia. Mice were anesthetized with 0.4 ml of a 15% nembutal solution given intraperitoneally. The animals were then shaved and the site of operation disinfected with a 1:10,000 solution of Zepherin. To remove the spleen the mouse was strapped to a board and a dorsal incision was made. The spleen was exteriorized, tied off with cotton thread and severed. The incision was then closed with an 11 mm Michel wound clip. The same procedure was followed to separate the left kidney, except that a ventral incision was used. All animals survived the operative procedure.

To stimulate erythropoiesis 2 groups of 10 mice each were given a single subcutaneous injection of phenylhydrazine hydrochloride or acetyl phenylhydrazine (0.06 mg per g body weight) 2 days before infection. Three types of red cell were seen on the smears stained with brilliant cresyl blue: the mature cell with no granulation, the reticulocyte with its deep blue granules, and mature cells with toxic granulation. The granular material in the toxic cells from animals treated with phenylhydrazine hydrochloride resembled that found in reticulocytes but stained a pale green. The cells from the acetyl phenylhydrazine-treated animals often showed a homogeneous disc in the center of the erythrocyte which was also pale green. These toxic cells were easily differentiated from reticulocytes on the basis of staining.

EXPERIMENTAL

Experiment 1. Splenectomy and nephrectomy of mice 2 days before infection with P. berghei.—The results of this experiment are shown in figure 1. Splenectomized animals exhibited a consistently lower parasitemia after the 6th day of infection than either nephrectomized mice or the unoperated controls. There was little difference in the parasitemia of the latter two groups. Surgery appeared to have no noticeable effect on the blood of uninfected mice as total red cell counts and percentage reticulocytes were well within the range for normal animals. It should also be noted (fig. 1) that there are no differences in the time of development or in the severity of anemia in the three infected groups. The percentage of reticulocytes is about the same for the unoperated controls as it is for the nephrectomized animals, while in the splenectomized mice reticulocytosis never attains the level seen in control groups. Surgery did not affect the survival time of the animals and differences in survival time are not statistically significant. Unoperated control animals survived a mean of 16.40 ± 0.57 days, nephrectomized controls survived a mean of 16.10 ± 0.54 days, and splenectomized mice survived a mean of 16.60 ± 0.49 days.

Experiment 2. Treatment of mice with phenylhydrazine hydrochloride or acetyl phenylhydrazine 2 days before infection with P. berghei.—Mice treated with phenylhydrazine hydrochloride or acetyl phenylhydrazine 2 days before infection showed higher parasite counts than control animals, as illustrated in figure 2. At the time of infection the host animals were somewhat anemic, having an average red cell count of 8,220,000 red blood cells per cu mm and an average reticulocytosis of 25%. Massive destruction of red cells begins earlier in the treated animals than in the untreated controls, but the rate of destruction is parallel for both groups. The percentage of reticulocytes is consistently higher in the treated parasitized group than in the untreated controls, but for the first 7 days of infection considerably lower than in the uninfected treated animals. The administration of these drugs did not affect the survival time of the animals and differences in survival time are not statistically

3. Taliaferro, W. H. and Taliaferro, L. G. 1940, J. Infect. Dis. **66**: 153–165.

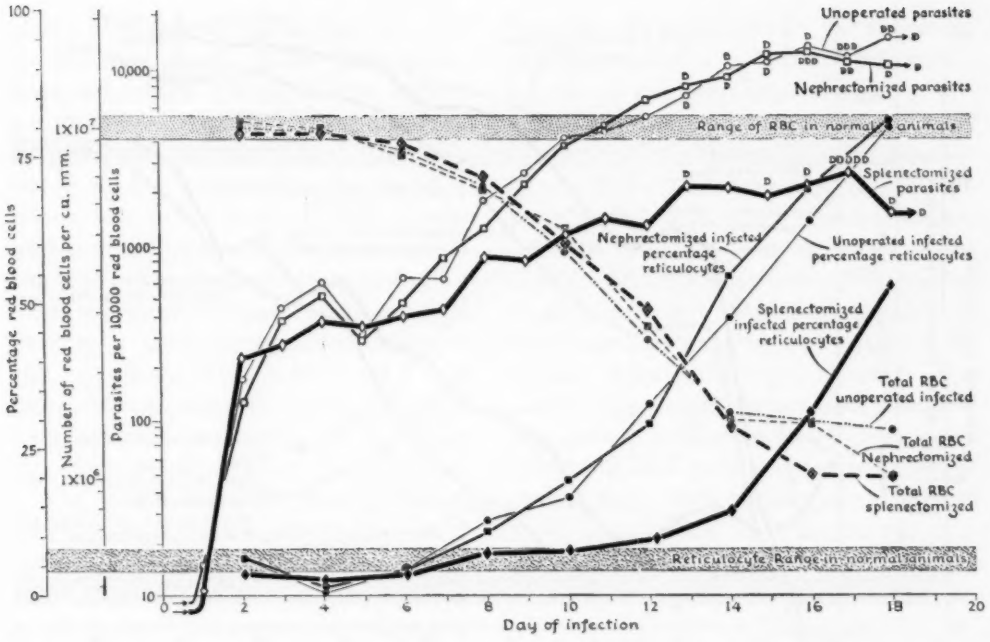
Unoperated parasites
Nephrectomized parasites
Range of RBC in normal animals
Splenectomized parasites
Nephrectomized infected percentage reticulocytes
Unoperated infected percentage reticulocytes
Splenectomized infected percentage reticulocytes
Total RBC unoperated infected
Total RBC Nephrectomized
Total RBC splenectomized
Reticulocyte Range in normal animals
Percentage red blood cells
Number of red blood cells per cu. mm.
Parasites per 10,000 red blood cells
Day of infection

FIG. 1.—The course of parasitemia and changes in the total red blood cell counts and percentage of reticulocytes in mice splenectomized or nephrectomized 2 days before infection and in unoperated control animals. D indicates the death of one mouse.

significant. Control animals survived an average of 15.75 ± 0.35 days, phenylhydrazine hydrochloride-treated animals survived an average of 15.70 ± 0.62 days, and acetyl phenylhydrazine-treated mice survived an average of 15.10 ± 0.57 days.

DISCUSSION

P. berghei infections in rats and voles[4–8] which have apparently been spontaneously cured may be caused to relapse, often fatally, by removing the spleen of the host. Splenectomy, in this case, is known to result in a breakdown of the immune mechanisms of the host. After complete cure rats are refractory to infection with the homologous strain of the parasite for about 205 days.[5] However, rats splenectomized during this period may be reinfected with the parasite and, although the peripheral blood is positive for about 220 days, residual immunity is able to prevent the death of the animals. When rats are splenectomized before infection[4] a more rapid and fatal disease results.

In contrast to the above, splenectomy of mice prior to infection results in a lowered parasitemia with no change in the survival time. Paradoxically, removal of the spleen enhances the innate immunity of the mouse by preventing the formation of large amounts of cells suitable for parasite penetration. Thus, the animal is deprived of tissue which is ordinarily somewhat erythropoietic and which has the potential of becoming

4. Galliard, H. and Lapierre, J. 1951, Bull. Soc. path. exot. **44**: 185–194.
5. Corradetti, A. 1950, Riv. Parassit. **11**: 201–209.
6. Fabiani, G., Vargues, R., Grillet, P. and Clausse, J. 1951, Compt. rend. Soc. de biol. **145**: 1131–1134.
7. Rodhain, J. 1951, Ann. Soc. Belge Med. Trop. **31**: 289–296.
8. Zuckerman, A. and Yoeli, M. 1951, J. Infect. Dis. **89**: 130–142.

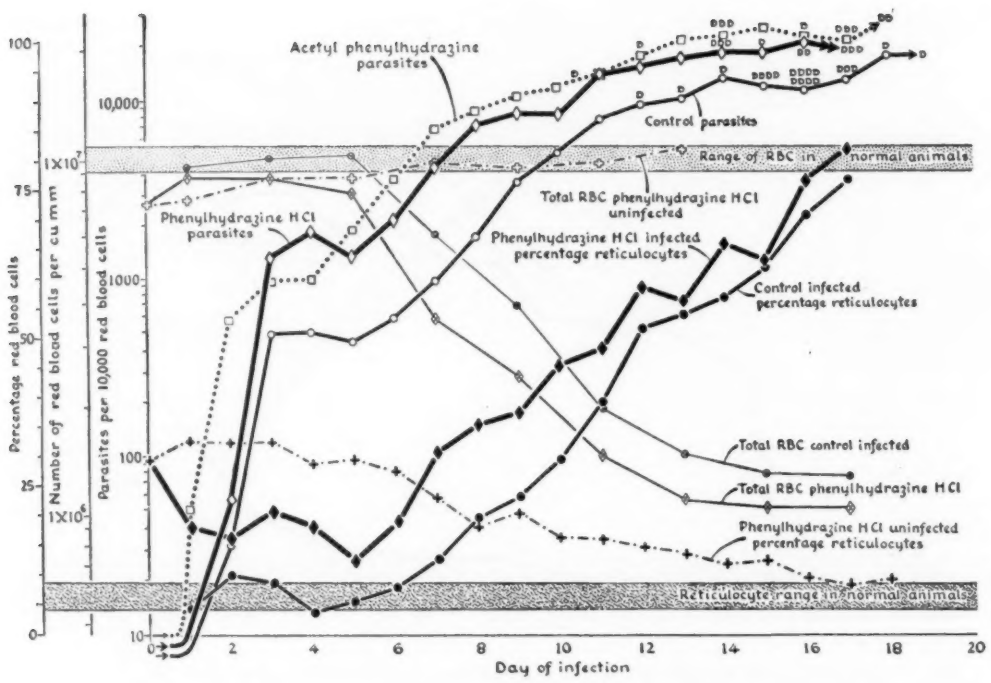
Acetyl phenylhydrazine parasites
Control parasites
Range of RBC in normal animals
Total RBC phenylhydrazine HCl uninfected
Phenylhydrazine HCl parasites
Phenylhydrazine HCl infected percentage reticulocytes
Control infected percentage reticulocytes
Total RBC control infected
Total RBC phenylhydrazine HCl
Phenylhydrazine HCl uninfected percentage reticulocytes
Reticulocyte range in normal animals
Percentage red blood cells
Number of red blood cells per cu mm
Parasites per 10,000 red blood cells
Day of infection

FIG. 2.—The course of parasitemia and changes in the total red blood cell counts and percentage of reticulocytes in controls and in phenylhydrazine hydrochloride or acetyl phenylhydrazine treated animals. D indicates the death of one mouse.

almost wholly so. The only large mass of tissue left to supply red cells is the bone marrow. It alone is incapable of achieving the level of production reached when the spleen is present. This is reflected by both the intensity of parasitemia and intensity of reticulocytosis seen in splenectomized infected animals (fig. 1), which are appreciably lower than those reached by either nephrectomized or unoperated infected controls. Furthermore, although anemia in infected splenectomized mice develops at the same time and rate as in the infected controls, the reticulocyte response is not as great and parasitemia is not as intense.

On the other hand, anemia induced by phenylhydrazine hydrochloride or acetyl phenylhydrazine and its attendant reticulocytosis results in a more severe infection than that seen in untreated animals (fig. 2). Previous studies on the effects of phenylhydrazine hydrochloride on malarial infections have led to conflicting reports. Hewitt[9] noted a marked increase of parasitemia in canaries infected with *Plasmodium relictum* and related this increase to the preference of the parasites for the younger red cells. Later it was shown that phenylhydrazine fed to infected chicks and ducks in their diet resulted in an inhibition of *Plasmodium gallinaceum* and *Plasmodium lophurae*.[10] Rigdon, Micks, and Breslin[11] have been able to cure *Plasmodium knowlesi* in the monkey with this drug, and more recently[12] have

9. Hewitt, R. 1937, J. Parasitol. **23**: 428–434.
10. Wiselogle, F. Y. 1946, Survey of Antimalarial Drugs, 1941–1945, Ann Arbor, J. W. Edwards, p. 870.
11. Rigdon, R. H., Micks, D. W. and Breslin, D. 1951, Am. J. Hyg. **52**: 308–316.
12. Rigdon, R. H., Micks, D. W. and Breslin, D. 1953, Tr. Roy, Soc, Trop, Med. & Hyg. **47**: 70–76,

shown that treated ducks infected with *P. lophurae* have a lower parasitemia than untreated ducks. These investigators suggest that the cured infection in the monkey is related to changes produced in the hemoglobin by the drug which are detrimental to the growth and multiplication of the parasite. In the duck they believe that phenylhydrazine injures the nuclei of the red cells, causing a subsequent degeneration of the cytoplasm. This is followed by the liberation of parasites which are unable to enter other red cells and are taken out of the blood stream by phagocytosis.

The results reported here agree with those seen in canary infections with *P. relictum*.[9] However, it must be kept in mind that at no time was any attempt made to treat the mouse infections with these drugs. Both phenylhydrazine hydrochloride and acetyl phenylhydrazine were administered 2 days before infection and then only to stimulate erythroid hyperplasia. The drugs apparently had no effect on the mechanisms of acquired immunity. The drop in parasite numbers noted in untreated animals[2] on the 5th day of infection was present and the survival time of the treated mice was unaffected. There was, however, a reduction in innate immunity. The treated animals were infected at a time when they exhibited hyperplastic erythroid tissue and a reticulocytosis. Parasitemia, therefore, was able to proceed at a higher level than in control animals because initially the host was supplying immature red cells at a greater rate than in normal animals. It is also interesting to note that the percentage of reticulocytes is lower in the phenylhydrazine-treated mice which were infected than in those which were uninfected for the first 6 days after the initiation of infection. This may be explained by the retention and agglutination of parasitized erythrocytes in the deep organs.[2]

The present results show that changes in the erythroid tissue of mice directly affects the number of parasites in the peripheral blood. Mice splenectomized prior to infection were deprived of potential erythropoietic tissue. In response to the infection they were unable to reach the reticulocyte level attained by intact animals and parasitemia was accordingly lower. Other animals having anemia and reticulocytosis prior to infection, due to the administration of drugs, were able to support a higher level of infection than untreated controls. Therefore, with an increase of reticulocytes, an increase in parasite numbers is evident. This indicates that *P. berghei* prefers the immature red cell or reticulocyte and that the level of parasitemia depends on the ability of the erythropoietic tissue of the host to supply these cells.

SUMMARY

Mice were splenectomized 2 days prior to infection or treated with phenylhydrazine hydrochloride or acetyl phenylhydrazine. After the 6th day of infection animals lacking spleens exhibited a lower parasitemia and lower reticulocyte count than the nephrectomized and unoperated controls. Drug-treated animals maintained a higher parasite count and more intense reticulocytosis through the course of infection than did control animals.

These results are discussed in relation to changes in erythroid tissue which govern the ability of the host to supply substrate for the parasite and in relation to the parasites' preference for immature erythrocytes.

THE EFFECT OF CORTISONE ON INFECTIONS WITH *PLASMODIUM BERGHEI* IN THE WHITE MOUSE

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Treatment of animals with cortisone or adrenocorticotropic hormone leads to a marked diminution in resistance to infection,[1] which, among other effects, may be related to the involution of lymphatic tissue. On this basis an especially vivid manifestation of lowered resistance might be predicted for malarial infections because of the established importance of the role of lymphatic tissue in supplying macrophages for defense.[2] The study of Schmidt and Squires[3] on infections with *Plasmodium cynomolgi* in the rhesus monkey supports this hypothesis. These investigators found that repeated injections of cortisone, during the primary attack, leads to a post-crisis intensification of parasitemia, or when administered during latency produces recrudescences reminiscent of the effects of splenectomy. These results are ascribed to the involution of lymphatic tissue and to the subsequent inability of the host animal to produce a quantity of lymphocytes sufficient to meet the defense requirement for a constant supply of new macrophages. Redmond[4] showed that *Plasmodium relictum* infections in pigeons treated with cortisone acted in a like manner. Later, Findlay and Howard[5] found that mice infected with *Plasmodium berghei* showed a more intense parasitemia and a decreased survival time when cortisone was administered, and also suggest this to be due to an inhibition of the cellular response of the host. In contrast to the work cited above the present results show that when cortisone is administered to CF1 mice infected with *P. berghei*, parasite counts are considerably lower than in control animals, and a statistically significant increase in survival time is noted.

MATERIALS AND METHODS

CF1 female mice (19 to 21 g) obtained from Carworth Farms were used in all experiments. Cortisone acetate was administered by the subcutaneous route. When necessary, this pharmaceutical was diluted with sterile physiological saline solution. Parasite counts, reticulocyte counts, and total red cell counts were made daily according to the methods described in an earlier paper.[6]

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The author wishes to express appreciation to Dr. Robert M. Lewert for his advice and assistance throughout the course of this work.

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1. Editorial. 1951, New Eng. J. Med. **245**: 75–77.
2. Taliaferro, W. H. and Mulligan, H. W. 1937, The Histopathology of Malaria with Special Reference to the Function and Origin of the Macrophages in Defence, Indian Medical Research Memoir, no. 29, Calcutta, Thacker, Spink & Co., Ltd.
3. Schmidt, L. H. and Squires, W. 1951, J. Exper. Med. **94**: 501–520.
4. Redmond, W. B. 1952, Proc. Soc. Exper. Biol. & Med. **79**: 258–261.
5. Findlay, G. M. and Howard, E. M. 1952, Nature **169**: 547.
6. Singer, I. 1954, J. Infect. Dis. **94**: 159–163.

EXPERIMENTAL

Experiment 1. Administration of 0.25 mg of cortisone daily to mice infected with P. berghei.—Mice infected with *P. berghei* were given 0.25 mg of cortisone daily, started 2 days before infection and administered throughout the course of infection. The results of this experiment are shown in figure 1. In the treated animals parasitemia is consistently lower and anemia and its attendant reticulocytosis develop at a slower rate than in untreated control animals. Infected control mice survived an average of 15.70 ± 0.65 days, while cortisone-treated, infected animals lived 21.70 ± 1.33 days—a statistically significant difference.

Representative animals were sacrificed on the 11th day of infection and various tissues were removed and prepared for examination. The reactions to infection in the cortisone-treated animals resemble those in the controls but appear to be retarded. The spleens are similar in size and appearance to those of untreated animals sacrificed on the 5th day of infection.[7] Although there is a marked increase in both erythro- and lymphopoietic tissue, mitoses are rare and lymphorrhexis is not apparent. The white pulp is predominately made up of medium lymphocytes and some large lymphocytes; depletion of small lymphocytes is evident (plate I, fig. 4). The spleens of cortisone-treated, uninfected animals appear smaller than normal spleens. There is no evidence for depletion and the splenic nodules are inactive (plate I, fig. 1 and 2). In addition, there is markedly less erythropoietic tissue than in the normal spleen. In the infected animals treated with cortisone, agglutination of parasitized erythrocytes is apparent in the sinuses and

7. Singer, I. 1954, J. Infect. Dis. In press.

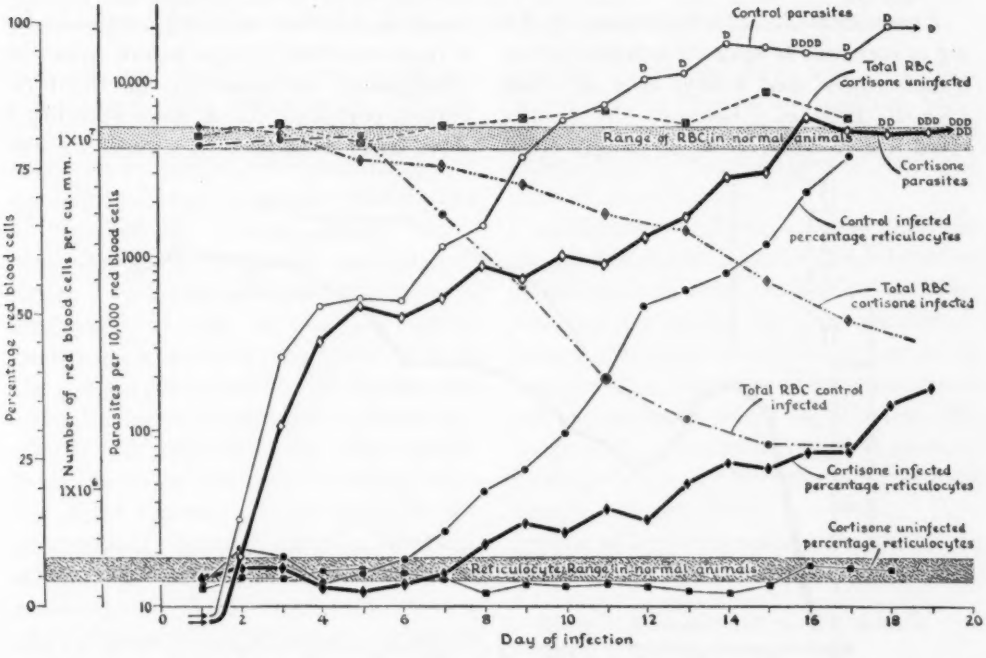

Control parasites
Total RBC cortisone uninfected
Range of RBC in normal animals
Cortisone parasites
Control infected percentage reticulocytes
Total RBC cortisone infected
Total RBC control infected
Cortisone infected percentage reticulocytes
Cortisone uninfected percentage reticulocytes
Reticulocyte Range in normal animals
Percentage red blood cells
Number of red blood cells per cu. mm.
Parasites per 10,000 red blood cells
Day of infection

FIG. 1.—The course of parasitemia and changes in the total red blood cell counts and percentage reticulocytes in controls and in mice treated with 0.25 mg of cortisone daily. D indicates the death of one mouse.

small blood vessels, and phagocytosis is largely restricted to the transitional zone of the white pulp. In the uninfected treated animals the macrophages of the spleen have ingested material which is transluscent and golden in color.

The lymph nodes of infected animals treated with cortisone are completely inactive and depleted, while those of the uninfected controls are inactive but not depleted (plate I, fig. 5 and 6). In the livers of infected treated animals phagocytosis is readily apparent and is restricted to the Kuppfer cells. In plate I, figures 7 and 8, sections of livers of treated infected and infected mice are compared. There is practically no cellular infiltration in the liver sinusoids of the infected treated animals, while in untreated mice sacrificed on the same day there are foci of both erythroid and lymphoid cells in the sinusoids. This is probably a reflection of the activity of the spleen.

Experiment 2. Administration of 5.0 mg of cortisone to mice 20 minutes before, 6 hours after, and 4 days after infection with P. berghei.—Because the results cited above differed so strikingly from those of Findlay and Howard,[5] the course of treatment followed by these investigators was applied to CF1 mice. It is apparent in figure 2 that their results were not confirmed. After the 4th day of infection the course of parasitemia is lower in the treated animals than in the untreated controls and is contiguous with the curve of infection described in experiment 1 (fig. 1) for mice receiving 0.25 mg of cortisone daily. The survival time of the treated animals was not significantly different from that of the untreated controls. The mice which received cortisone lived an average of 16.20 ± 0.67 days, while the controls survived 15.75 ± 0.35 days.

Experiment 3. Administration of 5.0 mg of cortisone to 2 groups of mice for 8 consecutive days starting 2 days before or 6 days after infection.—The results of this experiment are illustrated in figure 3. The animals were divided into 4 groups of 10 each. One group was untreated, another received cortisone for 8 days starting 2 days before infection (designated cortisone-1), the third received cortisone for 8 days starting 6 days after infection (designated corti-

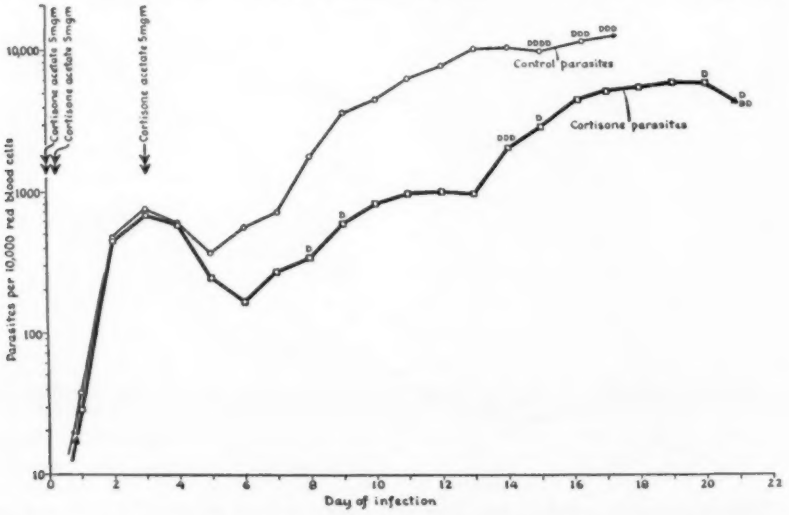


FIG. 2.—The course of parasitemia in controls and in mice treated with 5.0 mg of cortisone 20 minutes before, 6 hours after, and 4 days after infection. D indicates the death of one mouse.

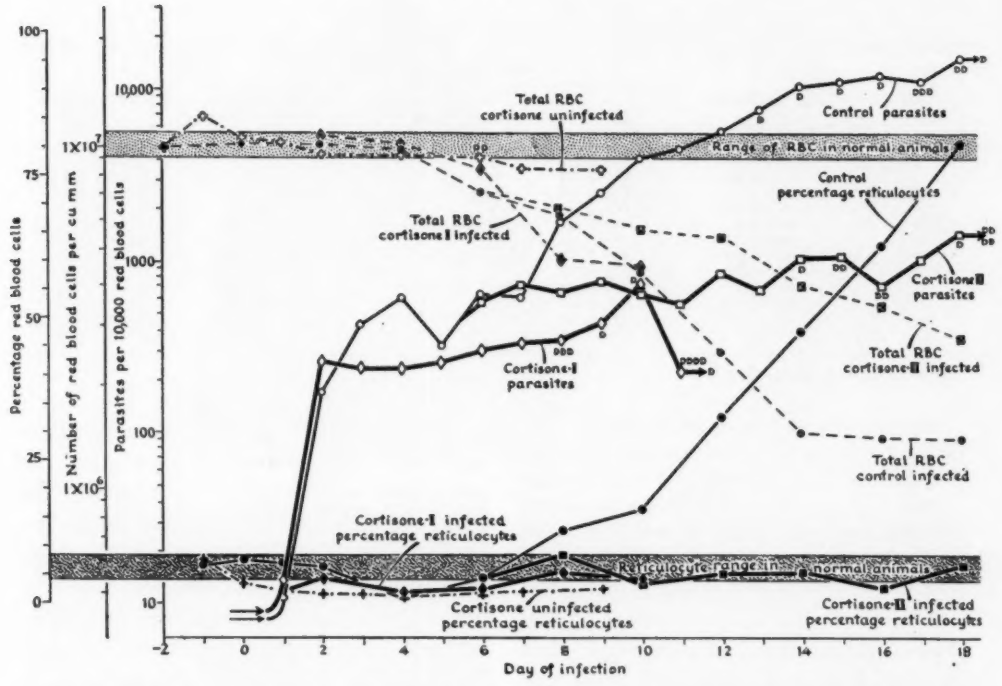

Percentage red blood cells
Number of red blood cells per cu mm
Parasites per 10,000 red blood cells
Total RBC cortisone uninfected
Control parasites
Range of RBC in normal animals
Control percentage reticulocytes
Total RBC cortisone I infected
Cortisone II parasites
Cortisone-I parasites
Total RBC cortisone-II infected
Total RBC control infected
Cortisone-I infected percentage reticulocytes
Reticulocyte range in normal animals
Cortisone uninfected percentage reticulocytes
Cortisone-II infected percentage reticulocytes
Day of infection

FIG. 3.—The course of parasitemia and changes in the total red blood cell counts and percentage reticulocytes in controls and in mice treated for 8 consecutive days with 5.0 mg of cortisone. The first injection was administered to the group designated cortisone-I 2 days before infection and to the group designated cortisone-II on the 6th day of infection. D indicates the death of one mouse.

sone-II), and the fourth was uninfected but treated with cortisone for 8 days. After the second day of infection the cortisone-I group exhibited a lower parasitemia than infected controls and a reticulocyte count which never rose above the range for normal animals. Two days after the initiation of treatment of the cortisone-II group parasitemia in these was lower than in the control group and terminally showed a total increase of only 600 parasites per 10,000 red blood cells. In other words, from the 6th to the 18th day of infection the total number of parasites in the cortisone-II group merely doubled, while it increased about 25 times in the control group. The reticulocyte count in the cortisone-II group never rose above the range seen in uninfected normal animals but was always above that seen in uninfected cortisone treated animals; anemia developed less rapidly in the treated group than in the controls. With this dose of cortisone 50% of the control animals were dead 11 days after the start of treatment.

Experiment 4. The effect of cortisone on an anemia induced by the administration of phenylhydrazine hydrochloride.—The results described in the previous experiments indicated that cortisone retarded anemia and depressed reticulocytosis due to malarial infection. To test this observation in another manner, animals having a phenylhydrazine-induced anemia were given cortisone. Five groups of five mice each were treated as follows:

Group 1: No treatment, normal controls
Group 2: 0.25 mg cortisone daily
Group 3: 0.25 mg cortisone daily; 0.06 mg per g body weight of phenylhydrazine hydrochloride, given in a single dose, 2 days after

TABLE 1.—*Percentage of reticulocytes.**

Day	No treatment	0.25 mg cortisone daily			Phenylhydrazine
			Phenylhydrazine given 2 days after cortisone	Phenylhydrazine given 2 days before cortisone	
1	4.44±0.06	5.28±0.04			10.72±0.20
2	5.92±0.06	2.58±0.03			17.82±0.19
3	8.10±0.11	1.52±0.03	1.74±0.03	27.00±0.12	26.84±0.23
4	6.96±0.06	1.48±0.03		16.36±0.13	27.44±0.22
5	7.04±0.06	1.62±0.03	4.38±0.06	8.16±0.06	32.44±0.26
6	7.30±0.09	4.28±0.18		3.74±0.04	24.28±0.24
7	6.66±0.09	2.20±0.13	6.29±0.14	4.36±0.05	18.00±0.18
8	7.60±0.08	3.86±0.19		4.98±0.04	18.12±0.30
9	6.60±0.12	3.40±0.02	5.86±0.09	6.58±0.08	18.00±0.22
10	6.50±0.09	3.70±0.05		6.76±0.06	16.10±0.19
11	7.10±0.09	3.20±0.06	4.80±0.06	6.54±0.01	14.60±0.14
12	7.60±0.07	2.80±0.15		6.28±0.03	13.80±0.21

* Average with standard error.

the start of cortisone administration

Group 4: 0.06 mg per g body weight of phenylhydrazine hydrochloride given in a single dose; 0.25 mg cortisone daily started 2 days after the administration of phenylhydrazine

Group 5: 0.06 mg per g body weight of phenylhydrazine hydrochloride given in a single dose

The percentage reticulocytes and total red blood cell counts are shown in tables 1 and 2. Animals treated with cortisone alone showed statistically significant lower reticulocyte counts and generally higher red cell counts than did normal animals. When phenylhydrazine was administered 2 days after the initiation of cortisone treatment the total red cell counts were lower than in those animals receiving cortisone alone but were appreciably higher than in mice given only phenylhydrazine. The reticulocyte counts showed the inverse, being higher than in the cortisone-alone animals but markedly lower than in those which had received phenylhydrazine alone. When cortisone was started 2 days after exposure to phenylhydra-

→

PLATE I

FIG. 1.—Cross section of the spleen of an uninfected CF1 mouse. ×14.

FIG. 2.—One-half the cross section of the spleen of a mouse sacrificed on the 11th day of infection. ×14.

FIG. 3.—Cross section of the spleen of a mouse which had been treated with cortisone for 13 days. Note that the size is smaller than that of an untreated animal (fig. 1) and that there is no depletion of white pulp. ×14.

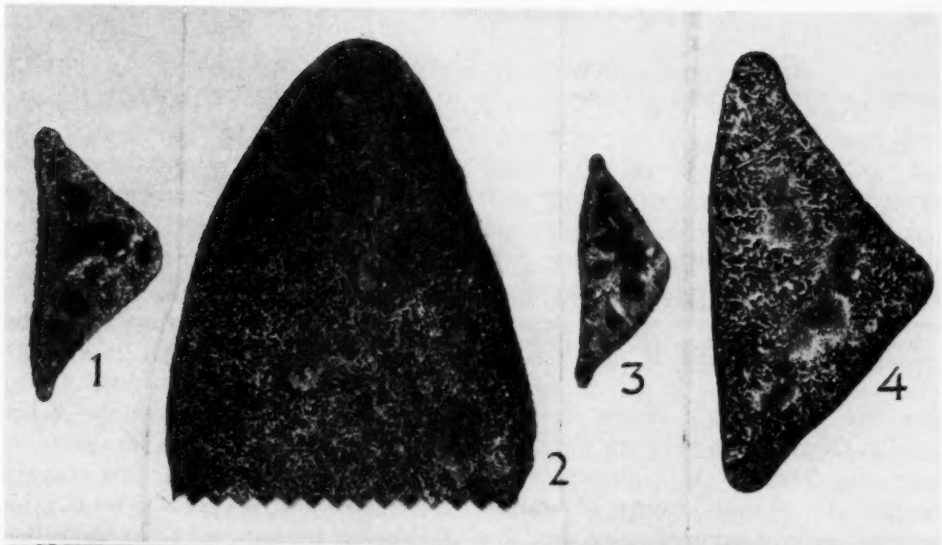
FIG. 4.—Cross section of the spleen from a mouse with an 11-day-old infection which had been treated with cortisone for 13 days. Note that the reaction to infection appears retarded when compared with the untreated infected mouse spleen seen in figure 2. It is not as large, does not show as much erythropoiesis and the white pulp, though depleted, takes up a greater area. ×14.

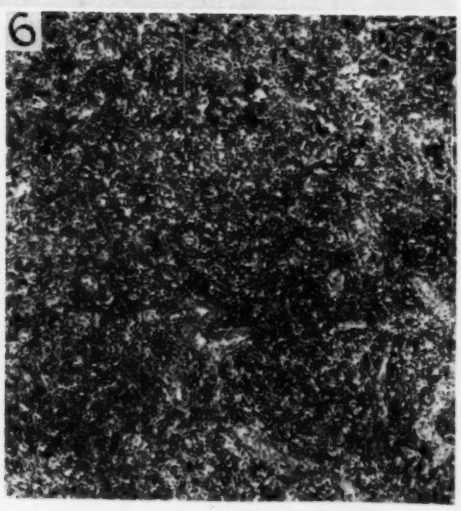
FIG. 5.—The cortex of an inguinal lymph node of an uninfected mouse which had been treated with cortisone for 13 days, showing neither depletion of small lymphocytes nor lymphopoietic activity. ×90.

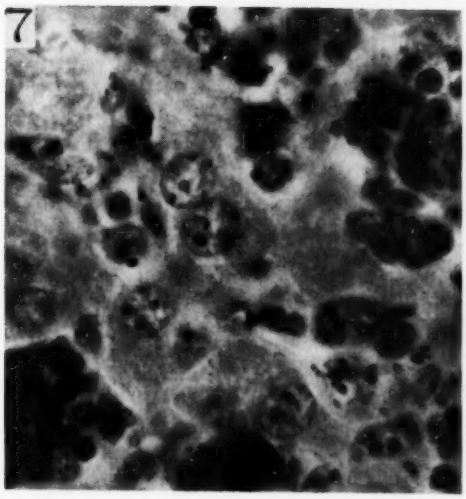
FIG. 6.—The cortex of an inguinal lymph node of a mouse sacrificed on the 11th day of infection after treatment with cortisone for 13 days, showing a depletion of small lymphocytes and no lymphopoiesis. ×90.

FIG. 7.—Section of the liver of a mouse sacrificed on the 11th day of infection, showing a group of erythropoietic cells in a sinusoid (lower left hand corner) and a nest of lymphoid cells in a sinusoid (upper right hand corner). Note the presence of hematogenous macrophages. ×840.

FIG. 8.—Section of the liver of a mouse sacrificed on the 11th day of infection after 13 days of cortisone treatment, showing a lack of infiltrating cells and that phagocytosis is restricted to the Kuppfer cells. ×840.







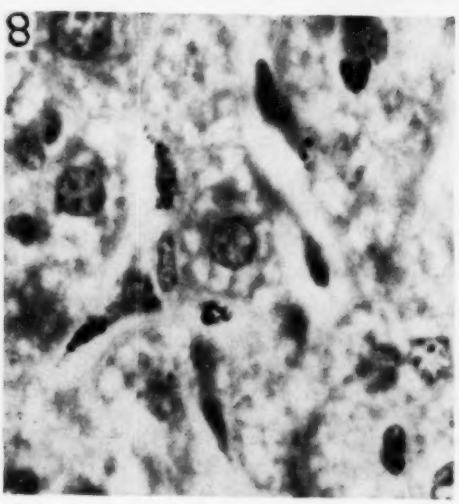


PLATE I

TABLE 2.—*Total red cell counts.**

Day	No treatment	0.25 mg cortisone daily			Phenylhydrazine
			Phenylhydrazine given 2 days after cortisone	Phenylhydrazine given 2 days before cortisone	
1	9.30±0.17	11.73±0.19			8.70±0.25
3	10.65±0.42	11.60±0.22	10.68±0.57	9.13±0.36	8.22±0.42
4	10.53±0.51	11.45±0.25	10.01±0.32	9.74±0.44	8.95±0.23
6	11.14±0.33	9.56±0.68	9.99±0.38	8.74±0.89	8.87±0.21
8	11.37±0.13	11.47±0.15	11.61±0.46	9.34±0.54	9.63±0.18
10	10.96±0.39	12.07±0.26	10.36±0.31	9.56±0.64	9.27±0.27
12	10.50±0.13	12.40±0.25	10.53±0.41	10.71±0.73	9.74±0.38

* Average red cell count given in millions per cu mm of blood with standard error.

zine the reticulocyte count dropped rapidly. One day after the start of cortisone treatment the reticulocyte count was 10% lower than in mice which had received only phenylhydrazine, and by the second day was well within the range for normal animals. However, it did not approximate the low level in animals receiving only cortisone.

DISCUSSION

The present results derived from experiments on CF1 mice are similar to those reported by Overman et al[8] for *Plasmodium knowlesi* infections in *Macacus mulatta* which were treated with a lipo-adrenal extract (compound E), and the opposite of those previously cited for *P. cynomolgi* infections in *Macacus rhesus*[3] and for *P. berghei* infections in white mice of an undefined strain.[5] The former investigators ascribe the lowered parasitemia and the increased survival time of treated animals to the re-establishment of functional proportions of the "salt-and-water-balance" and the "carbohydrate active" types of steroids of the adrenal cortex. Malarial infections are reported to cause directional changes in ion movements (Na and K) to and from cells. Similar changes occur in normal animals after the prolonged administration of desoxycorticosterone acetate (DCA, the "salt-water-balance" hormone). The administration of lipo-adrenal extract ("carbohydrate-active" hormone) to infected animals is said to have reversed the effects of malarial infection on ion movement in the cells of the host. By inference then, the administration of this hormone enhanced the innate immunity of the animal and resulted in lower parasite counts and increased survival times.

The present data do not permit interpretation of the results on this basis. Two avenues of conjecture are open: Cortisone may inhibit the growth and multiplication of the parasites or it may affect the host in such a manner that the infected animal is unable to supply suitable substrate for the maintenance of the plasmodium. The former possibility can be discarded on the basis of the reports mentioned earlier on *P. berghei* in white mice and on *P. cynomolgi* in monkeys. Previous studies[6,9] indicate that the degree of parasitemia seen in infections with *P. berghei* in white mice of the CF1 strain is dependent on the functional state of the erythropoietic tissue of these animals, and that when increased numbers of reticulocytes are being supplied, parasite counts are proportionately increased. In this case then, the lowered parasitemia and increased survival time of treated animals were considered to be due to a partial inhibition of hyperplasia of

8. Overman, R. R., Bass, A. C., Davis, A. K. and Golden, A. 1949, Am. J. Clin. Path. **19**: 907–917.

9. Singer, I. 1953, J. Infect. Dis. **92**: 97–104.

erythroid tissue. It is interesting to note here that Lowenstein et al[10] had previously found that ACTH produces a partial remission of bone marrow in pernicious anemia, and Palmer et al[11] have shown that cortisone prevents anemia in rats previously given anti-rat red cell serum. To reinforce this contention in another manner, a phenylhydrazine-induced anemia in CF1 mice was treated with cortisone. In this case cortisone prevented great erythroid activity, as reflected by reticulocyte counts (table 1). This further indicates that the action of cortisone enhances innate immunity by inhibiting the proliferation of erythropoietic tissue and depriving the parasite of suitable cells for penetration.

The disparity in these results with those of previously cited authors is due to differences in the degree of response. The experimental mice of Findlay and Howard are probably distinctly different physiologically from the inbred CF1 strain. In the CF1 mouse with anemia the spleen is apparently capable of usurping the function of the bone marrow. In this respect the response of the mouse resembles that of the chicken to *Plasmodium gallinaceum* infections more than it does that of other mammals to infections with various malarial organisms.[12]

Studies on tissues from cortisone-treated mice sacrificed on the 11th day of infection show basically similar reactions to cortisone-treated monkeys infected with *P. cynomolgi*.[3] In uninfected control animals the expected lymphatic involution,[13] lymphocytolysis,[13] and decrease in macrophage activity[14] did not occur. The spleens are slightly decreased in size (plate I, fig. 3) and show no activity in the secondary nodules. The germinal centers of lymph nodes are quiescent and there is no depletion of small lymphocytes from the cortex of the nodes (plate I, fig. 5). In the infected animals which received cortisone the usual tissue response[6] to infection appeared to be retarded. Lymph nodes were depleted and inactive (plate I, fig. 6). In the spleen the white pulp still made up a large part of the organ and erythropoietic tissue had not reached the proportions usually attained by the 11th day of infection (plate I, fig. 4). Despite the relatively hyperplastic state of the organ, mitoses were infrequent when compared to controls. Phagocytosis per se by the fixed and free macrophages in the spleen and liver appeared to be enhanced in animals receiving cortisone. An examination of plate I, figures 7 and 8, shows that the Kuppfer cells in the livers of cortisone-treated animals contain heavier clumps of malarial pigment and appear to be more active than in control mice. On the other hand, the quantity of macrophages is considerably less in the cortisone-treated mice. This may be explained by the profound reduction in the proliferative activity of the lymphatic tissues under the influence of the hormone. In other words, the administration of cortisone resulted in a reduction of the major source of supply of new macrophages essential for defense against malaria.

In conclusion, cortisone appears to have similar but not as marked effects on CF1 mice as those seen with X-ray administration.[8] However erythro- and

10. Lowenstein, L., Shapiro, L. and Browne, J. S. L. 1951, Proc. Sec. Clin. ACTH Conf. **1**: 426–437.
11. Palmer, J. G., Cartwright, G. E. and Wintrobe, M. M. 1951, Proc. Sec. Clin. ACTH Conf. **1**: 438–453.
12. Taliaferro, W. H. J. Infect. Dis. In press.
13. Dougherty, T. F. 1952, Physiol. Rev. **32**: 379–401.
14. Spain, D. M., Molomut, N. and Haber, A. 1950, Science, **112**: 335–337.

lymphopoietic activity is not destroyed but is only inhibited. Depressed erythropoiesis is reflected in both lowered parasitemias and reduced reticulocyte counts, while depressed lymphopoiesis becomes evident on tissue examination. There are inactive germinal centers in the lymphatic tissues and a paucity of hematogenous macrophages in the organs usually involved in cellular defense.

SUMMARY

The administration of cortisone to mice with infections of *Plasmodium berghei* resulted in lowered parasitemias and reduced reticulocyte counts. Tissue examination revealed a decreased quantity of macrophages and a probable enhancement of phagocytic activity on the part of the fixed and free macrophages. These results are related to an inhibition, by the hormone, of proliferative activity in both erythro- and lymphopoietic tissues and to the subsequent inability of the host to supply both suitable substrate (reticulocytes) for the parasites and lymphocytes as a source of macrophages for its own defense.

CHANGES IN HOST-CELL PREFERENCES IN MALARIAL PARASITES AND THEIR RELATION TO SPLENIC RETICULAR CELLS

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Knowledge of the interaction of malarial parasites and their host cells is essential to an understanding of many of the questions concerning the course of infection, the immunology, the histopathology, and chemotherapy of malarial infections. One of the most basic factors in these interrelations is that which determines what type of cell will be invaded by the parasite at a particular time and under particular circumstances. Enough is now known about the changes which occur in the type of host cells inhabited during the course of sporozoite-induced infections, as well as in infections induced by the inoculation of erythrocytic and exoerythrocytic stages, to indicate that they are not due to chance alone. Not only are there definite patterns of behavior of the host-cell affinities of certain species of malarial parasites in a given host, but certain laboratory strains become altered so that their affinities depart from the usual pattern of host-cell affinities. I shall concern myself in the following statements with descriptions of some of these patterns of behavior of some avian malarial parasites and with the possible role of certain splenic cells in influencing this behavior.

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OBSERVATIONS AND DISCUSSION

The inability of sporozoites to enter erythrocytes is now well established in many species of malarial parasites. In the more intensely studied species, such as *Plasmodium cathemerium* (Porter, 1942) and *Plasmodium gallinaceum* (Huff and Coulston, 1944), it has been established that a few generations of pre-erythrocytic stages live in cells of the lymphoid-macrophage system and that there is then a shift to the endothelial cells of the veins and capillaries. Concomitant with or subsequent to the invasion of the endothelial cells the erythrocytes are invaded. There are variations on this general pattern probably caused by differences in the hosts and the antecedent history of the strains. At the present time it is impossible to determine the degree of interdependence of the parasites which produce the concomitant erythrocytic and exoerythrocytic infections. In these species of avian parasites we know that erythrocytic stages may arise from exoerythrocytic stages and vice versa. However, there is increasing evidence to indicate that succeeding generations of parasites show a greater tendency to select the same type of host cell than to transfer to other types of host cell. The extent to which the parasites shift their preferences for different cell types is difficult to determine because: (1) in order to enumerate the various forms of parasites it is necessary to sacrifice the host, and (2) between the time of inoculation of a particular form and the time when parasites become numerous

enough to be found in the inoculated animal, many events have taken place to confuse the result.

When sporozoites of any of several of the avian species of *Plasmodium* (*gallinaceum*, *relictum*, *cathemerium*, *lophurae*, or *fallax*) are introduced, either by mosquito bite or artificially by syringe and needle, into the skin of a susceptible bird some of them develop in the macrophages of the local injection site, others are destroyed by heterophil leucocytes, and still others are carried away by the lymph or blood (Huff and Coulston, 1944; Coulston and Huff, 1947; Huff, 1951; Huff, Coulston, Laird, and Porter, 1947; and Huff et al, 1950). At present we are unable to determine whether the sporozoites which develop in the local site or those carried to other parts of the body are the more important in establishment of an infection. Although it is possible in the case of the parasites which develop locally to follow their development from very early stages to fully grown schizonts and to determine in what type of host cells they are developing, it is not possible to follow the early development of the sporozoites which enter the circulation. Even when small animals and extremely large numbers of sporozoites are employed, one is often unable to locate the parasites during the first generation of their development (Huff, 1951). In the few instances in which occasional parasites have been located during the first two generations of development following the intravenous inoculation of large numbers of sporozoites, their location has been the reticular cells of the Schweigger-Seidel sheaths of the spleen (Huff & Coulston, 1944).

Since it was known that exoerythrocytic stages could be derived from erythrocytic stages we decided to attempt to find out the time and place of this change. We therefore inoculated large doses of infected erythrocytes intravenously into uninfected animals. Since the inoculum in these cases was so large as to produce an immediate, heavy parasitemia, there was not the same difficulty in discovering parasites in the inoculated animals from the moment of inoculation as was the case in sporozoite-induced infections. Intensive histological search has, however, failed to reveal the presence of any exoerythrocytic stages in the organs of chicks (26), ducklings (7), pigeons (4), or canaries (5) inoculated with erythrocytic stages of *P. fallax;* nor in canaries (15) inoculated with *P. relictum;* nor in chickens (7) inoculated with *P. lophurae.* The following organs were examined: adrenal gland, bone marrow, brain, heart, kidney, liver, lung, spleen, thymus, thyroid, and gonad. However, in the following hosts similarly inoculated, exoerythrocytic stages were demonstrated: chicks with *P. gallinaceum* (7 out of 40), turkeys with *P. fallax* (9 out of 19); turkeys with *P. lophurae* (6 out of 19); and canaries with *P. cathemerium* (1 out of 13). Although the animals were sacrificed at periods of time from 3 to 19 days following inoculation, exoerythrocytic stages were found no earlier than the 4th or 5th days after inoculation. The significant fact in these findings is that *the first exoerythrocytic stages seen in animals in which the infections had been induced by the inoculation of erythrocytic parasites were always seen in the reticular cells of the Schweigger-Seidel sheaths of the spleen.*

In our earlier studies on sporozoite-induced infections we were prone to think that the reason why the first observed exoerythrocytic stages were in the spleen was that this organ is the most effective filter for removing the sporozoites from the blood. The failure to discover exoerythrocytic stages in the other organs of these birds and in

none of the organs of a large proportion of the animals was attributed to the very great dispersal of the parasites over the relatively huge volume of the host. Such an explanation is, of course, not acceptable for the absence of exo-erythrocytic stages from the organs of animals having infections induced by the intravenous inoculations of huge numbers of erythrocytic stages. A characteristic of well-developed exo-erythrocytic infections is the prevalence of these forms in the endothelial cells of the venules and capillaries. However, the parasitemia which existed from the moment of inoculation of the erythrocytic stages made possible the continuous, close association of erythrocytic parasites and the very endothelial cells which would eventually be infected. In spite of this close association, there was no earlier entry of the endothelial cells than in sporozoite-induced infections.

It is obvious that we are here dealing with changes in the affinities of the parasites for certain host cells and that after such changes occur they tend to be inherited by subsequent generations of the parasites. I use the word "inherited" in its broadest sense rather than implying that the changes are governed by genes. Explanations of such changes must be sought in the known mechanisms for transfer of such properties from one cell to its progeny. Among such known mechanisms are (1) true mutations and (2) the Dauermodifikationen of Jollos (1921) or the "transformations" of Sonneborn (1951a and b).

To attempt to explain the changes by means of any hypothesis involving mutations would require the admission that such mutations were an invariable prerequisite to the establishment of an infection in its natural manner and that inverse mutations were continually occurring in infections induced by the inoculation of infected erythrocytes. Although this places a large burden upon such a hypothesis it should not yet be discarded as a possibility. The *transformations* produced in antigenic types of *Paramecium* by Sonneborn (1951) by immune serum and various physical and chemical agents appear to be more nearly comparable phenomena. If the changes in host-cell selectivity on the part of *Plasmodium* are transformations, they appear to concern changes in metabolism reflected in ability to subsist in different types of host cell rather than in alterations in the substances which act antigenically when introduced into vertebrate animals, as is the case of *Paramecium*.

Until such time as the mechanism of these changes can be determined I shall avoid the use of either of the terms "mutation" or "transformation." Moreover, the term "modification" carries the implication that the change is not inherited and, therefore, is to be avoided. For the present I intend to refer to these changes in the host cell preferences by malarial parasites as "conversions."

Regardless of the explanation of the intrisic mechanism for the control of these conversions, the experimental evidence to date points toward some special function of the splenic reticular cells in initiating the change. It would be unwise at present to deny the possibility that other host cells may take part in the process, but the histological evidence indicates that the reticular cells of the Schweigger-Seidel sheaths probably play the most important role in initiating the conversions in the parasites.

Since orderly patterns exist in the conversions in parasites which occur during the course of infections induced either by sporozoites or by erythrocytic stages in the individual avian hosts, the logical approach to an explanation

would be a correlation between the conversions in the parasites and the changes induced in the host by the infection. The physiopathological changes in the host include: (1) acquired immunity, (2) anemia, and a variety of other less well defined conditions. Inasmuch as the earlier conversions occur often before the development of any appreciable anemia it does not seem probable that the anemia could act as an initiating cause. If, however, one postulates that immune substances initiate the conversions in the parasites the splenic reticular cells would appear to be possible sites for their production. Such a hypothesis would not only relate the phenomenon to the transformations of antigenic structure in *Paramecium* through the action of immune substances but would also be in accord with the widely held view of immunologists that the spleen is an important site of antibody production. If one should adopt this hypothesis it would not necessarily follow that the reticular cells of the Schweigger-Seidel sheaths are the unique sites of antibody production or the only cells capable of initiating conversions in the parasites. It might well be that in these splenic cells are combined in high degree (1) the ability to produce antibodies and (2) a suitable environment for the development of the parasite. Other cells such as macrophages, endothelial cells, and erythrocytes might provide one of these conditions but not the other.

SUMMARY

In infections of *Plasmodium gallinaceum* induced in chicks by the intravenous inoculation of either sporozoites or erythrocytic stages the first exoerythrocytic stages to be observed were located in reticular cells of the Schweigger-Seidel sheaths. Exoerythrocytic stages of *Plasmodium lophurae*, *Plasmodium fallax*, and *Plasmodium cathemerium* were likewise first observed in similar cells, respectively, of the turkey and of the canary, following the intravenous inoculations of huge numbers of erythrocytic stages. Since the circulating parasites had access to the endothelial cells from the moment of their inoculation but did not invade these cells until after they had produced exoerythrocytic stages in the splenic reticular cells, it appears likely that they were changed in some way by their residence in the splenic cells. The changes effected in the parasites are referred to by the noncommittal term "conversions," to avoid implying that they are known to be either mutations or transformations. Although the mechanism of these changes is not yet understood, the most plausible hypothesis appears to be that antibodies produced within the splenic reticular cells bring about conversions in the potentialities of the parasites for living in in the different types of host cells, these conversions probably being comparable to the changes induced by antibodies in the antigenic characteristics of *Paramecium*.

REFERENCES

Coulston, F. and Huff, C. G. 1947, The morphology of cryptozoites and metacryptozoites of *Plasmodium relictum* and the relationship of these stages to parasitemia in canaries and pigeons. J. Infect. Dis. **80**: 209–217.

Huff, C. G. 1951, Observations on the pre-erythrocytic stages of *Plasmodium relictum*, *P. cathemerium*, and *P. gallinaceum* in various birds. J. Infect. Dis. **88**: 17–26.

Huff, C. G. and Coulston, F. 1944, The development of *Plasmodium gallinaceum* from sporozoite to erythrocytic trophozoite. J. Infect. Dis. **75**: 231–249.

Huff, C. G., Coulston, F., Laird, R. L. and Porter, R. J. 1947, Pre-erythrocytic development of *Plasmodium lophurae* in various hosts. J. Infect. Dis. **81**: 7–13.

Huff, C. G., Marchbank, D. F., Saroff, A. H., Scrimshaw, P. W. and Shiroishi, T. 1950, Experimental infections with *Plasmodium*

fallax Schwetz isolated from the Uganda tufted guinea fowl *Numida meleagris major*. J. Nat. Mal. Soc. **9**: 307–319.

Jollos, V. 1921, Experimentelle Prostistenstudien. I. Untersuchungen über Variabilität und Vererbung bei Infusorien. Arch. f. Protist. **43**: 1–222.

Porter, R. J. 1942, The tissue distribution of exoerythrocytic schizonts in sporozoite-induced infections with *Plasmodium cathemerium*. J. Infect. Dis. **71**: 1–17.

Sonneborn, T. M. 1951a, Beyond the gene—Two years later. From Science in Progress, G. A. Baitsell, editor, Yale Univ. Press.

———. 1951b, The role of the genes in cytoplasmic inheritance. Chap. 14 in Genetics in the 20th Century, L. C. Dunn, editor, New York, Macmillan.

STUDIES ON THE HELMINTH FAUNA OF ALASKA

XX. THE HISTOGENESIS OF THE ALVEOLAR LARVA OF ECHINOCOCCUS SPECIES

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The occurrence of the larval stage of *Echinococcus granulosus* (Batsch, 1786) in a microtine rodent, *Clethrionomys rutilus* Pallas, on Bering Island was reported by Barabash-Nikiforov (1938) and Afanas'ev (1941). Rausch and Schiller (1951) reported this cestode from tundra voles, *Microtus oeconomus* Pallas, collected on St. Lawrence Island. Further observations have shown that it occurs also in *C. rutilus* in the same location. Both species of rodents have a wide circumboreal distribution.

Continued study of this cestode, involving experimental infections of various mammals and comparison with the larval development of *E. granulosus*, has shown it to be specifically distinct. It is differentiated on the basis of the host-species occurrence of the larval stage, and on larval form. Detailed study of the adult cestode did not disclose morphological characters which consistently differentiate it from *E. granulosus*. However, on a morphological basis the adult differs clearly from all material examined excepting specimens from foxes (*Alopex* and *Vulpes*) taken on Nunivak Island and along the Arctic Coast of Alaska. Further study of this latter material is required (Rausch, 1952 and 1953).

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The laboratory infections of the animals used in this work have been established jointly by the writer and Mr. E. L. Schiller. The writer particularly wishes to express appreciation of the assistance of Miss R. V. Sacressen, who prepared the histological material and drew the figures.

Alveolar hydatid disease in man is endemic in southern Europe and in Asia, but the life cycle of the cestode involved has never been completely worked out. The Eurasian form may be identical with that occurring on the Bering Sea islands; however, confirmation will depend upon experimental infections using Eurasian cestode material. Alveolar hydatid disease, apparently first recognized by Virchow (1855), has been studied by several investigators. The work of Dévé and Posselt has been especially important, and the problem has been reviewed in detail by Dardel (1927). Although the writer wishes to point out the possibility that the two forms are conspecific, it is not considered appropriate to review here the voluminous literature concerning alveolar hydatid disease.

It is the purpose of this paper to present a detailed account of the histogenesis of the larval stage of echinococcus species from St. Lawrence Island, based on material derived from laboratory infections.

MATERIAL AND METHODS

The natural final host of echinococcus species on St. Lawrence Island is the arctic fox, *Alopex lagopus* L., but sledge dogs are also frequently infected by ingesting rodents harboring larvae. Experimental infections were initially established by feeding infected vole liver to captive arctic foxes. Voles and other rodents were infected at first by feeding gravid segments taken from foxes and dogs autopsied in the field, but the entire cycle was later established in the laboratory.

For the present work, gravid segments of

echinococcus species taken at autopsy from foxes were used to infect rodents. Immediately after the death of the fox, the intestine was opened and the contents washed out in cold tap water. The cestodes were concentrated by pouring off the supernatant fluid and were freed of debris by repeated washings. The presence of eggs was confirmed by use of a dissecting microscope.

Rodents to be infected were anesthetized with ether, and the eggs administered by means of a glass tube directly into the stomach. Entire gravid segments were introduced, and the number of eggs administered to a single animal ranged from about 400 to 2000.

This study was based on experimentally induced infections in laboratory-reared field voles, *Microtus pennsylvanicus* Ord, obtained originally from the eastern United States. For observations on the earlier developmental stages, subadult voles of uniform size were used. No differences in susceptibility or in larval development correlated with age of the host animal have been observed, and both subadult and adult animals have been used in the study of the later stages. Other rodents have been experimentally infected (viz., *Ondatra*, *Peromyscus*, *Clethrionomys*, and other species of *Microtus*), and comparative observations were made. Only a small amount of human material was available for study.

It was not possible to obtain for comparison early developmental stages of *E. granulosus* larvae, but infective cysts were obtained from naturally infected moose (*Alces*). Materials from an Australian wallaby (*Wallabia*) and from man were also compared.

Infected rodents were killed at desired intervals, and tissues were fixed in 10% formalin or in formalin-acetic acid-alcohol solution. Tissues were embedded by the paraffin method and routinely stained with hematoxylin-eosin and Weigert's iron hematoxylin with van Gieson's picro-acid fuchsin solution counterstain. Special stains were applied in some cases.

RESULTS

The primary locus of infection in rodents was, without exception, the liver. Larval development in this organ, beginning the first day after infection, is described below for the common field vole, *Microtus pennsylvanicus*.

At 20 hours.—Necrotic foci up to 140μ in diameter, involving a relatively few hepatic cells, are visible. There is some leukocytic infiltration, with macrophages and a few polymorphonuclear neutrophils. The larvae were not observed.

At 26 hours (fig. 1).—Macroscopically, no changes were visible.

In liver sections, larval loci are disclosed by diffuse leukocytic infiltration into irregularly-shaped areas of necrotic cells. Such cells take a faint stain and show disruption of the nuclear elements. Polymorphonuclear neutrophils are present in good numbers, but more numerous are irregularly-shaped leukocytes which may have their origin in the von Kupffer cells of the sinusoids. The larvae, located with difficulty, are situated singly in the masses of necrotic liver cells. They measure about 15μ in diameter, and consist of spherical masses of cells with prominent, dark-staining nuclei, and pale, acidophilic cytoplasm. The total number of leukocytes is relatively small, and the greatest density occurs in the immediate vicinity of the larvae.

At 48 hours.—The liver is hyperemic, and minute white foci are visible macroscopically.

There is little change in larval size over the previous day. The zone of necrotic liver cells is much larger, and a peripheral zone of degenerating cells is seen around it. Leukocytes are more numerous and are infiltrated over a greater area. It is evident that the larvae have penetrated the walls of the interlobular veins in entering the lobules. The path of migration is densely infiltrated by mononuclear leukocytes.

At 79 hours (fig. 2).—In sections, the larvae are seen as spherical to irregularly shaped vesicles up to 30μ in diameter. These vesicles are comprised of flat cells with prominent, dark-staining nuclei. The effect on the host tissue is increasingly severe, resulting in an ever enlarging zone of necrotic and degenerating liver cells around each larva. Imme-

diately around the larvae, polymorphonuclear neutrophils are predominant, but some macrophages are present. Mononuclear cells (apparently lymphocytes) are predominant peripherally and in the areas through which the larvae migrated when entering the lobules. Large numbers of leukocytes are now seen in the blood vessels.

At 96 hours.—Macroscopically, the larvae and surrounding tissue reaction have increased in size, and the liver is hyperemic.

There is some increase in the size of the larval vesicles, but this is not marked. The zone of dense leukocytic infiltration is usually 80 to 100μ thick, but the zone of degenerating hepatic cells may be much larger. Polymorphonuclear neutrophils comprise almost the entire leukocytic reaction at this time, although a few macrophages are visible. Giant cells are rarely observed.

At 140 hours.—The larval vesicles have increased in diameter up to 50μ. There is little change in the leukocytic barrier, but fibroblasts have appeared peripherally between the degenerating and normal tissue. There is perivascular infiltration of leukocytes involving the interlobular veins.

At 164 hours.—Macroscopically, the superficial foci have increased in size.

The larval vesicles have attained a diameter of as much as 70μ. Eosinophils are numerous in the vicinity of the larvae. Each larval vesicle is surrounded by a dense zone of leukocytes about 40μ thick. Beyond this, necrotic and degenerating hepatic cells form another zone, and the whole is surrounded by fibroblasts to a depth of about 20μ. Some fibrosis has taken place, and there is an abrupt transition from degenerating to normal liver cells. The path of larval migration is still infiltrated by leukocytes.

At 212 hours (fig. 3).—The primary vesicles show a slight thickening of the germinal layer. A large proportion of them contain secondary vesicles which have originated by endogenous budding, and secondary vesicles are being produced also by exogenous budding. In the latter cases, extensions from the germinal membrane invade the connective tissue adventitia, vacuolate, and increase in size. In such cases leukocytes (mainly polymorphonuclear neutrophils) surround the secondary vesicle, and persist as it increases in size. These cells are eventually trapped be-

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FIG. 1–8.—The symbols designate the following structures: NHC, normal hepatic cell; DHC, degenerating hepatic cell; GM, germinal membrane; NGM, nucleus of germinal membrane; PV, primary vesicle; ENV, larval vesicle of endogenous origin; EXV, larval vesicle of exogenous origin; DS, developing scolices; SGM, subgerminal membrane; CC, calcareous corpuscles; S, scolices.

FIG. 1.—Embryo at 26 hours after infection. From 10μ section stained with hematoxylin-eosin.

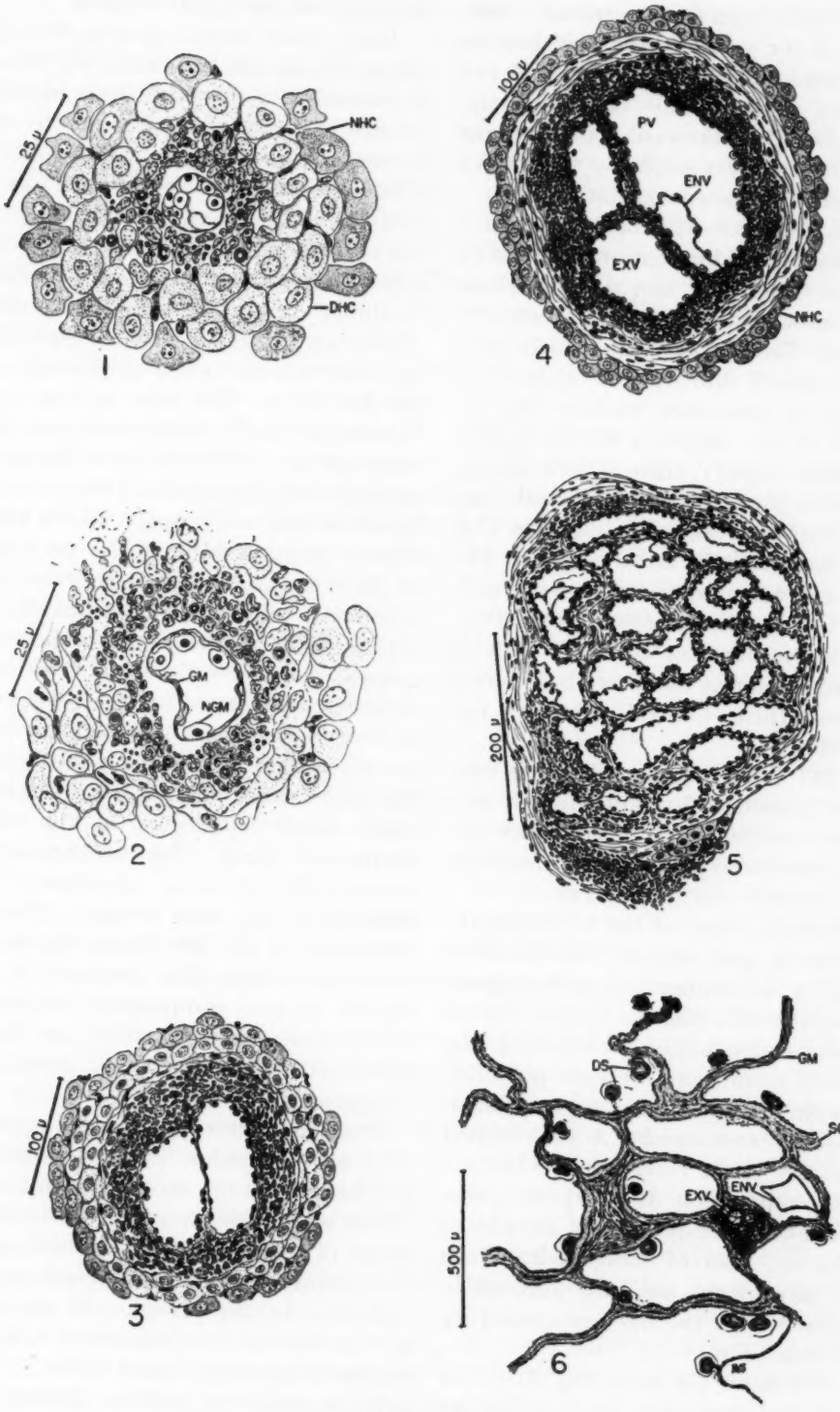
FIG. 2.—Primary larval vesicle at 79 hours after infection. From 10μ section, stained with hematoxylin-eosin.

FIG. 3.—Primary vesicle with secondary vesicle of exogenous origin at 212 hours after infection. From 6μ section stained with hematoxylin-eosin.

FIG. 4.—Larval mass, at 287 hours after infection, showing vesicles both of endogenous and exogenous origin. From 10μ section, stained with Weigert's hematoxylin and van Gieson's counterstain.

FIG. 5.—Alveolar form of larva at 336 hours after infection. The primary vesicle can no longer be identified, and both endogenous and exogenous development of secondary vesicles are taking place. From 6μ section, stained with Weigert's hematoxylin and van Gieson's counterstain.

FIG. 6.—Detail of larval vesicles at 45 days after infection. A few scolices are being produced, and both endogenous and exogenous development of secondary vesicles are continuing. From 10μ section stained with hematoxylin-eosin.


25 μ
NHC
DHC
1
100 μ
PV
ENV
EXV
NHC
4
25 μ
GM
NGM
2
200 μ
5
100 μ
3
DS
GM
SGM
ENV
EXV
500 μ
M
6

FIGURES 1–6

tween two layers of germinal membrane as the vesicles grow and become contiguous. Frequently the loci of two primary vesicles may be closely apposed, but these are easily differentiated from secondary vesicles by their thick connective tissue encapsulation.

At 234 hours.—Macroscopically, it is is possible to differentiate the larval vesicles from the surrounding tissue reaction when they are superficially located. Gross specimens which have been stained and cleared show that genesis of secondary vesicles has occurred in the majority of the larvae.

Microscopically, there is little change visible beyond continued growth and proliferation of secondary vesicles. Cellular characteristics are unchanged. The path of larval migration remains densely infiltrated by mononuclear cells. Perivascular infiltration by such cells is marked insofar as the interlobular veins are concerned. Central veins do not show this.

At 287 hours (fig. 4.)—There is continued growth and proliferation of secondary vesicles, and several may develop simultaneously in the connective tissue capsule of a single larva.

A tendency toward the later alveolar structure is now evident. There is little change in the arrangement and composition of the cells surrounding the larval masses. Immediately surrounding the germinal membrane are seen polymorphonuclear neutrophils, macrophages, and a few eosinophils. A well-formed connective tissue layer is next, bordering directly upon the hepatic cells. There is evidence of pressure atrophy of the latter. Foci of mononuclear cells occur irregularly and are apparently associated with the damage caused by larval migration.

At 336 hours (14 days) (fig. 5).—The liver is enlarged and the vesicles are prominent. The separate larval masses have a diameter of 500 to 1500μ.

Very rapid larval growth has occurred during the last two days. This is evidenced by a relatively great increase in size of the larval mass and by the presence of many secondary vesicles. The connective tissue capsule, previously dense and well-formed, has been disrupted, with the result that the stroma supporting the larval vesicles is in direct contact with the liver cells. There is a marked increase in vascularity. There is also some proliferation of the bile ducts. The zone of liver cells showing pressure atrophy is larger. The sinusoids are distended, and the entire organ is very hyperemic. The numerous larval vesicles are supported by a loose, fibrous stroma. Around the periphery of the mass, mononuclear cells are predominant, and have infiltrated the hepatic cells. Either singly or in small groups, hepatic cells have been surrounded and isolated by the leukocytes, so that necrotic and degenerating cells are numerous within the periphery of the larval mass. The stroma itself contains many leukocytes in the more peripheral parts. Polymorphonuclear neutrophils are most abundant, but eosinophils are also present. Toward the center of the larval mass the leukocytes are fewer. The presence of disrupted nuclear components and other debris indicates that they are being destroyed in the course of larval development.

There is little change in the appearance of the vesicles. There is, however, a thickening of the germinal membrane. There is no evidence yet of the development of a subgerminal ("laminated") membrane. Rapid larval growth is also indicated by the presence, at the edge of the stroma, of numerous isolated masses of germinal tissue which are the forerunners of new vesicles. There is less endogenous larval development, but

some of the larger vesicles show this process.

At 20 days.—The superficially located larvae, up to 3 mm in diameter, are seen as coalesced aggregations of translucent vesicles. The liver is hyperemic, and collateral circulation is developing. This is seen particularly as a proliferation of the superficial branches of the interlobular veins, with numerous anastomoses in the immediate vicinity of the vesicles.

Microscopically, there is increase in vesicle size. Pressure atrophy of the liver cells is particularly evident. The sinusoids are distended with erythrocytes, and there is marked dilatation of the central veins. There has been a reduction in the number of leukocytes. Polymorphonuclear neutrophils are abundant in the immediate vicinity of the vesicles, and mononuclear cells are still moderately numerous around the periphery. The germinal membrane is relatively thick, but there is no evidence of early scolex development. The subgerminal membrane is well developed. There is a narrow zone of fibroblastic proliferation, with numerous collagenous fibers.

At 27 days.—There is a continuing increase in vesicle size and number, with a corresponding increase in the size of the infected organ.

Sections show that almost all cellular elements of host-tissue origin have been eliminated from within the mass of vesicles. The larval mass has assumed an alveolar structure and is comprised of great numbers of contiguous and interconnected vesicles. There is no evidence of scolex formation. Outside the subgerminal layer is a very thin, but dense, connective tissue barrier separating the parasite tissue from the host tissue. All visible hepatic cells show pressure atrophy, probably resulting from the combined pressures of the rapidly growing vesicles and the greatly distended sinusoids.

At 34 days.—Atrophy of the hepatic cells is marked. There appears to be breaking down of the erythrocytes in the sinusoids. A thin zone of collagenous fibers separates the subgerminal membrane from the hepatic tissue. A very thin zone of leukocytes, mainly polymorphonuclear neutrophils, is found immediately adjacent to the subgerminal membrane. The germinal membrane is loosely arranged and is relatively thick. A few scolices are seen, in the early stages of development.

At 45 days (fig. 6).—Macroscopically, the size of the liver is greatly increased over normal. The vesicles show a tendency to become confluent.

There is continued growth of larval vesicles. Scolex development is progressing, but none is fully developed and infective. The remaining hepatic tissue is being destroyed.

At 50 to 150 days.—During this time there is a great increase in size of the infected liver. Large collateral blood vessels become prominent on the surface and the amount of visible liver tissue is becoming progressively less.

Microscopically, there is an increase in the amount of parasite tissue, with a progressive destruction of the remaining liver tissue. The branches of the portal vein are dilated, and the sinusoids of the remaining liver tissue are greatly congested. The central veins also show dilation, but not to the extent that perivascular necrosis results. Pressure atrophy of hepatic cells is general; there is also some necrosis resulting from the effect of toxic products elaborated by the parasites. During this time scolex production goes on rapidly; at first there are only a few scolices, widely scattered over the germinal membrane lining the vesicles, but later the vesicles are nearly filled by them. The vesicles are

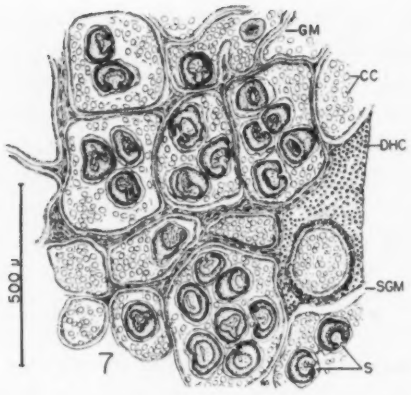


FIG. 7.—Structure of larval mass 5 months after infection. Infective scolices are abundant and little hepatic tissue remains.

not fluid-filled, as is characteristic of *E. granulosus*. The subgerminal membrane is well developed, averaging 10 to 12μ thick. It may, however, show undulations and thickened areas. Beneath it, a dense layer of connective tissue, from a few up to 100μ thick, surrounds individual vesicles and forms barriers between them and the remaining liver tissue. The importance of leukocytes becomes progressively less. Finally, only a few scattered polymorphonuclear neutrophils may be seen along the subgerminal membrane.

At 150 to 170 days (fig. 7 and 8).—The infected liver attains maximum size and death of the host may occur. The living animal is dyspneic and slow of movement. The infected liver may constitute two-thirds of the total weight of the animal. Nothing but parasite tissue may be visible, but the cut surface may show a few islets of liver cells still recognizable macroscopically as such.

Microscopically, the vesicles are filled with infective scolices. Calcareous corpuscles are abundant. There is a tendency toward a radial arrangement of the walls of the secondary vesicles, and it is usually possible to recognize units of vesicles which have developed from the original embryo. The individual vesicles are usually less than 10 mm in diameter. Sections show a follicular appearance of the larval mass, but there is a tendency for the aggregations of vesicles to become confluent; this is best seen in cleared, gross sections. The aggregations of vesicles which have originated from a single embryo contain few or no elements of host-tissue origin. In rare instances, there may be isolated remnants of hepatic cells or a few leukocytes. Frequently, however, such tissue elements are entrapped when the larval aggregations become confluent. These undergo destruction, and no normal hepatic tissue is visible. There is a reduction in the amount of connective tissue around the larval mass, as a result of the rapid larval growth. Apparently by this time the host body is no longer capable of utilizing its defense mechanisms.

Changes in the cellular components of the circulating blood.—Significant changes in the cellular composition of the circu-

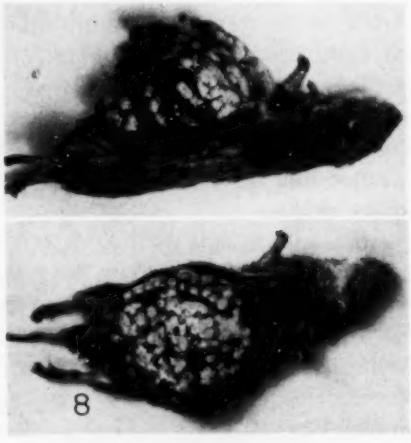


FIG. 8.—A heavily infected field vole killed in the terminal stages of disease 5 months after infection. The animal weighed 98.0 g; of this, the infected liver weighed 59.1 g, with a volume of 60 cc. The maximum normal weight for this species of vole rarely exceeds 60 g. The arrow indicates the position of the stomach and duodenum.

lating blood were not observed in smears stained with Wright's stain. Even in the terminal stages, about 5 months after infection, there was no eosinophilia.

Larval development in other rodent species.—Comparative study of infections in other species of microtine rodents failed to disclose any differences in larval development. In a few cases vesicles of unusual size (up to 20 mm) developed, along with those of more typical appearance, but this was also observed in the common field vole.

Metastatic spread of larvae.—In both natural and experimental infections of long duration, secondary foci of infection in the spleen, mesentery, peritoneum, etc. were frequently observed. These originated through metastasis. The dilatation of blood vessels resulting from the larval growth apparently is adequate to allow bits of larval tissue to pass through into the hepatic vein and thence into the systemic circulation. It is probable that such larval tissue originates as exogenous buds which gain entrance to the blood stream by perforation of a vessel wall.

Intra-abdominal larval development.—A very small quantity of germinal membrane containing a few scolices was injected through an 18-gauge needle into the abdominal cavity of a subadult vole. This animal, as a large, breeding male, was killed 160 days later. An ovoid mass of larval vesicles, measuring 10×15 mm, was found free in the abdominal cavity.

Microscopically, it was evident that development was not entirely normal. The mass consisted essentially of proliferated subgerminal membrane tissue, in which there were large calcified areas. Normal-appearing vesicles were present only peripherally, and these were lined with scolices which appeared to be completely developed and infective. No cellular components of host-tissue origin were recognized. It is indicated from this that the main function of the subgerminal membrane is supportive, and that it may proliferate extensively under some circumstances.

DISCUSSION

The intermediate stage of *E. granulosus* occurs mainly in perissodactyls and artiodactyls. Durie and Riek (1952) reported wallabies (*Thylogale* and *Wallabia*) to be important intermediate hosts in Australia. As far as the writer has been able to determine, however, rodents rarely harbor the intermediate stage of *E. granulosus*. Certain rodents (e.g., *Myocastor*) have been reported as harboring *echinococcus* larvae, but the species involved is probably not *E. granulosus* (Rausch, 1953). It is concluded that in this case host-species occurrence is significant, since it has not been possible to infect microtine rodents with *E. granulosis* in the laboratory. It cannot be considered that larval form is influenced by abnormal host-species occurrence.

Various authors have described the slow rate of development of *E. granulosus* larvae in sheep and swine. Dew (1925) reported a vesicle diameter of 40 to 50 mm in swine at the end of 3 months after infection. With a vesicle diameter of 100 mm there was no development of scolices at the end of 5 months. Turner et al (1937) rarely observed cysts 1.0 cm in diameter 11 or twelve months after infection of experimentally infected sheep. Earlier workers, including Dévé and Leuckart, obtained similar results. In Alaska, larval vesicles containing matured scolices in naturally-infected moose (*Alces*) rarely exceeded 60 mm in diameter. All North American specimens of *E. granulosus* larvae examined by the writer from naturally infected cervids have con-

sisted of simple, fluid-filled cysts surrounded by a dense connective tissue layer.

Dew (1925) observed that the larvae of *E. granulosus* become vesicular and somewhat enlarged at 14 days. The larvae of the St. Lawrence Island cestode are vesicular 3 days after infection. Dew found that the entire larval follicle, including the total tissue reaction, measured but 250 to 350μ at the end of 3 weeks. The aggregation of larval vesicles of the St. Lawrence Island form averages about 3 mm in diameter at 21 days. The thick connective tissue capsule surrounding the larval vesicle of *E. granulosus* is also indicative of slow growth. As mentioned earlier in this paper, no such well-developed connective tissue capsule is seen associated with the later larval stages of the St. Lawrence Island cestode.

The proliferation of secondary vesicles is essentially endogenous insofar as *E. granulosus* is concerned, or, at best, exogenous production is infrequent. The mechanism of secondary cyst formation has been reviewed by Dew (1926). He concluded that true exogenous development of secondary vesicles does not occur, and in this he disagreed with several earlier writers.

CONCLUSIONS

The larva of the St. Lawrence Island cestode attains its alveolar form by means of a rapid rate of proliferation of exogenous secondary vesicles. This also explains its ability to invade host tissue so rapidly, and with such a malignant effect. It is concluded, then, that ability in the St. Lawrence Island cestode to produce secondary vesicles exogenously constitutes the main difference between its larval development and that of *Echinococcus granulosus*.

REFERENCES

Afanas'ev, V. P. 1941, Parazitofauna promyslovykh mlekopitaiushchikh Komandorskikh Ostrovov. Uchenie Zapiski, Seriia biologicheskikh Nauk. **18**: 93–117.

Barabash-Nikiforov, I. 1938, Mammals of the Commander Islands and the surrounding sea. J. Mammal. **19**: 423–429.

Dardel, G. 1927, Das Blasenwurmleiden in der Schweiz, Bern, 105 pp.

Dew, H. R. 1925, The histogenesis of the hydatid parasite (*Taenia echinococcus*) in the pig. Med. J. Australia **1**: 101–110.

———. 1926, The mechanism of daughter cyst formation in hydatid disease. Med. J. Australia **1**: 451–460.

Durie, P. H. and Riek, R. F. 1952, The role of the dingo and wallaby in the infestation of cattle with hydatids (*Echinococcus granulosus* (Batsch, 1786) Rudolphi, 1805) in Queensland. Med. J. Australia **28**: 249–254.

Rausch, R. 1952, Hydatid disease in boreal regions. Arctic **5**: 157–174.

———. 1953, The taxonomic value and variability of certain structures in the cestode genus *Echinococcus* (Rud., 1801), and a review of recognized species. Commemoration vol. for Dr. G. S. Thapar. Lucknow, India. In press.

Rausch, R. and Schiller, E. L. 1951, Hydatid disease (Echinococcosis) in Alaska and the importance of rodent intermediate hosts. Science **113**: 57–58.

Turner, E. L., Dennis, E. W. and Berberian, D. A. 1937, The production of artificial immunity against hydatid disease in sheep. J. Parasitol. **23**: 43–61.

Virchow, R. 1855, Die multiloculäre, ulcerirende Echinokokkengeschwulst der Leber. Verh. phys.-med. Ges. Würzb. **6**: 84–95.

SOME RELATIONSHIPS BETWEEN LYMPHOCYTIC CHORIOMENINGITIS (LCM) VIRUS AND MICE

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The virus (LCM) which causes in man the disease lymphocytic choriomeningitis exists in nature as a parasite of the common mouse (*Mus musculus*, var.). The natural virus-mouse relationship permits survival of the parasite on terms that are very easy for the host; yet under circumstances producible artificially, the virus displays lethal capabilities in the mouse, and the mouse shows a capacity for complete suppression of virus, at least in a form demonstrable by the usual methods.

The human disease caused by this virus appears to be of limited importance as far as present knowledge goes, but further study of the variable effects of parasite and natural host upon one another offers an opportunity for increasing our knowledge of host-parasite relationships, a subject of fundamental significance to the control of infectious disease. This report discusses such relationships in terms of severity of infection, and of distribution and persistence of virus in various mouse tissues. It shows how such factors as the route of injection and the degree of immunity in the mouse have distinctive influences upon the pattern of virus survival and development.

The original papers of Armstrong et al, Rivers and Scott, Traub, Finlay et al, and other investigators describing the human disease and the characteristics of murine infections have been reviewed and documented by van Rooyen and Rhodes.[1] No general review appears necessary here, but specific references are made in the text where published observations have a direct bearing on the presentation, or where recognition of prior contributions is indicated.

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MATERIALS AND METHODS

LCM virus used in these studies was obtained from pooled blood of several mice trapped in houses in Washington, D. C., where human cases had been reported during the preceding year.* Infected brains used in the experiments were taken from mice of an intracerebral (i.c.) passage series originating from the pooled blood, unless otherwise indicated. This strain was maintained by continuous passage (0.05 cc of 10^{-3} brain emulsion) at 6- or 7-day intervals. Infected spleen, kidney, and bits of liver were obtained (unless otherwise stated) from mice of an intraperitoneal (i.p.) passage, made at 6- or 7-day intervals, using emulsions of these tissues pooled (0.25 cc of 10^{-3}).

Identity of the virus was established by (a) nature of illness in mice, (b) incubation period, (c) cross immunity with the original strain (Green) isolated by Armstrong,[2] and (d) pathology in mouse brain, as determined by Dr. James H. Peers of the National Institutes of Health.

All grinding of tissues was done by hand in a mortar; dilutions were made with buffered physiological saline solution, without centrifuging except where stated.

Mouse and rat serums were from pools of blood obtained by cardiac puncture, and unless used immediately were preserved in the refrigerator; 2 drops of penicillin solution (100 units/cc) and 2 drops of streptomycin solution (100 μg equivalents/cc) were added to each lot (3 to 5 cc) of serum before storage.

1. Van Rooyen, C. E. and Rhodes, A. J. 1948, Virus Diseases of Man, ed. 2, New York, Thomas Nelson and Sons.

*I am indebted to Dr. Charles Armstrong for records of these human infections, and to laboratory technician T. R. Williams for trapping the mice (1948).

2. Armstrong, C. and Lillie, R. D. 1934, Pub. Health Rep. **49**: 1019–1026.

TABLE 1.—*Recovery of LCM from perfused tissues of infected mice.*

Exp. no.	Type of infection	Tissue tested	Deaths per 5 mice inoculated intracerebrally with tissue suspension in indicated logarithmic dilutions						
			3	3.5	4	4.5	5	5.5	6
1	In utero	Brain		4	3	2	2	0	
		Liver		1*	1*	5	3	3	
		Spleen		3*	5	4	5	2	
		Kidney		5	5	5	5	0	
2	In utero	Brain	5	5	5	5	5	1	
		Liver	5	5	5	5	3	0	
		Spleen	2*	5	5	4	5	5	
3	In utero i.c. passage, 6th day postinoculation	Brain	5	5	4	3	0	0	0
		Brain	5	4	5	5	5	5	1

* Survivors developed nonfatal illness.

Tests for viremia in mice were made on blood taken by cardiac puncture, diluted approximately to 10^{-1} in buffered physiological saline. Each mouse used for assaying viremia received 0.05 cc of this mixture intracerebrally, plus 0.25 cc intraperitoneally, except where stated otherwise.

Tests for virus in tissues other than blood were made by intracerebral inoculations with appropriate saline emulsions, 0.05 cc of 10^{-3}, except where otherwise indicated.

In specific studies on patterns of LCM virus development, mice were given intracerebral injections of 0.05 cc of 10^{-2} emulsion of infected brain which had been centrifuged at 1200 r.p.m. for 10 minutes. Brains of these mice, to be tested for virus at suitable intervals after inoculation, were ground in saline, 1:30, and centrifuged; inoculation into test mice was made intracerebrally with 0.05 cc of supernatant.

Mice were NIH white Swiss stock, unstandardized as to age, weight, or sex. In each experiment where comparisons were involved, mice were taken from a single batch. Most mice used for testing and routine transfers weighed 18 to 20 g and were between 4 and 5 weeks old at the time of initial use.

St. Louis encephalitis (SLE) virus was from the original (Freeman) strain.[3] It was processed in a manner similar to LCM brain virus. In mice tested for SLE virus development patterns, the original inoculum intracerebrally was 1:500, instead of 10^{-2} as for LCM.

Influenza virus used for immunization was the PR-8 strain, in allantoic fluid from chick embryos,* given to mice intraperitoneally, 0.50 cc of 1:50 dilution in saline.

Neutralization tests with LCM and SLE were done in the manner described by Wooley, Armstrong and Onstott[4] and by Wooley and Armstrong,[5] respectively, except that virus dilutious were as indicated in the tables. Neutralization against PR-8 virus was tested in the manner described by Eddy.[6,7]

Variations in severity of infection

The existence of LCM virus in nature has long been known to depend upon murine in utero transmission.[8,9] During the present studies a characteristically mild infection was maintained in this manner through 12 mouse generations; viremia was found in every one of 255 mice selected randomly from more than 150 litters, and virus was present in considerable quantities in perfused vital organs (table 1). Yet all mice in the series were indistinguishable in external appearance and behavior from uninfected ones of the same stock.

An essentially inapparent infection can be produced artificially by subcutaneous injection of LCM brain emulsion.[2] During the present studies hun-

3. Muckenfuss, R. S., Armstrong, C. and McCordock, H. A. 1933, Pub. Health Rep. **48:** 1341–1343.

*I am indebted to Dr. Bernice Eddy Wooley for this material, and for assistance with influenza neutralization tests.

4. Wooley, J. G., Armstrong, C. and Onstott, R. H. 1937, Pub. Health Rep. **52:** 1105–1114.
5. Wooley, J. G., and Armstrong, C. 1934, Pub. Health Rep. **49:** 1495–1504.
6. Eddy, B. E. 1947, J. Immunol. **57:** 195–202.
7. Eddy, B. E., and Ward, T. G. 1952, J. Bact. **63:**341–352.
8. Traub, E. 1936, J. Exper. Med. **64:** 183–199.
9. Traub, E. 1939, J. Exper. Med. **68:** 801–817.

TABLE 2.—*Recovery of LCM from tissues (nonperfused) of mice of routine passage series on 6th postinoculation day.*

Passage series	Tissue tested	Route of injection for test	Mice dying per 5 injected with logarithmic dilutions indicated											Illness noted in surviving mice
			3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	
i.c.	brain				5	5	5	5	5	5	5	5	4	
i.p.	brain				2	5	2	2	3	4	4	2		All mice quite sick
i.c.	l, s, k*	i.c.		5	3	1	1	0	0					None ill beyond dilution 5.5
i.p.	l, s, k			5	4	5	5	5	4	3	4	3	3	All sick
i.c.	brain		0	2	0	0	1	1						All definitely sick
i.p.	brain	i.p.	0	0	1	1	0	0						All definitely sick
i.c.	l, s, k		0	0										Mild, transitory sick
i.p.	l, s, k		2	3	3	1	3							All definitely sick

* Pooled bits of liver, spleen and kidney.

dreds of mice were so inoculated with emulsions of brains from the intracerebral passage series (0.25 cc of 1:50); detectable illness was absent or mild; in 133 mice whose records were analyzed there were only 2 deaths.

Mild or inapparent infections can be induced by intranasal instillation of virus,[2,10] but attempts to establish a strain by serial passage intranasally were unsuccessful during the present studies. Frank illness developed in mice after the usual emulsions of abdominal organs used for intraperitoneal passage had been given intranasally, but subsequent passage with lung emulsion produced no detectable illness. Mice of passages 2, 3, and 4 were later found immune to intracerebral challenge, but further passage produced neither illness nor immunity.

Another variety of inapparent infection sometimes developed during these studies in mice receiving intracerebral injections which failed to cause detectable illness, presumably because virus was present in small quantities. In 150 such mice noticed during miscellaneous experiments, 56 were subsequently immune to intracerebral challenge.

The lethal capabilities of LCM in mice become evident when virus is injected into the brain. Routine passage in the present studies with 10^{-3} brain emulsion killed all but 12 of a series of 500 mice. In titration tests, freshly harvested nonperfused brain from the intracerebral passage strain regularly killed all mice inoculated intracerebrally, in dilutions as high as $10^{-7.5}$ (table 2).

A severe but less fatal infection can be produced by injecting virus intraperitoneally.[10] In 500 mice used for routine passage by this route, with 10^{-3} emulsions of pooled liver, spleen and kidney, there were 192 survivors, all with chronic illness. Such emulsions given intraperitoneally produced illness in dilutions as high as 10^{-5} (table 2). The lower fatality rates from intraperitoneal injection appear attributable to route of inoculation and not to quantity of virus: emulsions of brains from the intracerebral series, producing high death rates when injected by the intracerebral route, caused illness with low fatalities when given intraperitoneally (table 2), even though the intraperitoneal dose was 5 times that given intracerebrally.

Variations in infection from inapparent to fatal were obtained by using blood diluted 10^{-1} in saline as the inoculum. Serial passage by subcutaneous injection (0.25 cc) was made 69 times: the mice almost never appeared ill, and among 200 there were only 27 deaths, of which 19 were attributed to cardiac puncture and removal of blood. In

10. Traub, E. 1936, J. Exper. Med. **63**: 533–546.

order to demonstrate viremia, each subcutaneous transfer was accompanied by inoculation of another set of mice intracerebrally (0.05 cc); when these sentinel mice became ill, transfer was made from their subcutaneously injected mates. Among 100 mice of the intracerebral series, 84 deaths were attributable to the infection. Serial passage was also made 92 times intravenously (0.25 cc): mice developed illness similar to that caused by intraperitoneal injection;[10] there were 226 deaths in 300 inoculations.

Subcutaneous injection of infected blood was accompanied in 24 mice by intracerebral inoculation of a thick suspension of cornstarch: 4 died of what appeared to be typical LCM infection. Cornstarch alone produced no effect in 18 mice. This method of converting an inapparent infection into a fatal one was the means by which Traub[13] initially demonstrated virus in his naturally infected mouse colony (using broth, not starch). In Traub's mice and in those reported here, the proportion of inapparent infections converted to fatalities was small.

It has been reported by earlier workers[11,12] that passage history of the virus has an effect upon the nature of the infection it can produce. In the present studies, there appeared to be a definite tendency for liver-spleen-kidney emulsions from the intraperitoneal series to produce more definite illness when given subcutaneously than any tissue emulsions from the intracerebral series. Brain emulsions from the intraperitoneal series seemed to produce less fatalities than those from the intracerebral series, with a tendency to chronicity suggestive of that seen in mice of the regular passage groups injected by the intraperitoneal route. Data available (table 2) are not sufficient to give unequivocable support to the impression that these differences are entirely attributable to different methods of virus passage, but they do not preclude such a hypothesis.

Distribution and persistence of virus in the mouse

In the mild or inapparent infection acquired in utero, LCM is distributed widely and persists indefinitely in the mouse.[14] In artificially induced infections, virus is often, though not always, demonstrable in a variety of mouse tissues; it has strong tendencies of persistence, but there are limitations.[8,12,14,15]

During the present studies, examinations of 136 inoculated mice for persistence of LCM indicated that the strains of virus tested can survive for more than 6 months (table 3), but failure to demonstrate virus in 24 of these mice (not specifically identified in the data tabulated) indicates that some of the mice achieved the ability to suppress their infections, at least so far as demonstrable virus was concerned.

TABLE 3.—*Recovery of LCM from mice at intervals after inoculation: summary of results from 136 tests.*

Tissue inoculum and route of injection in mice employed as host for virus survival	Recovery of virus from specified tissues of host mice: numerator is latest postinoculation day of virus recovery; denominator is latest day of trial		
	Brain	Liver, spleen, kidney	Blood
Brain—i.c.	80/135	102/135	6/135
Brain—s.c.	28/135	23/135	9/135
Liver, spleen, kidney—i.p.	79/193	193/193	193/193
Liver, spleen, kidney—s.c.	63/135	135/135	135/135
Blood—i.c.	102/102	102/102	Not tested

A group of mice were given 2 inoculations with infected brain emulsion, 10^{-2}

11. Traub, E. 1937, J. Exper. Med. **66**: 317–324.
12. Schwartzmann, G. 1946, J. Immunol. **54**: 293–303.
13. Traub, E. 1935, Science, **81**: 298–299.
14. Traub, E. 1936, J. Exper. Med. **63**: 847–861.
15. Traub, E. 1938, J. Exper. Med. **68**: 229–250.

TABLE 4.—*Unsuccessful attempts to stimulate suppression of viremia in mice infected in utero with LCM*

Exp. no.	Number of mice	Injections given in attempts to stimulate LCM suppression	Number of tests for viremia per mouse, 10 to 30 days after each injection—all positive
1	15	LCM virus, routine i.c. passage strain, 10^{-3} i.c.—3 to 5 injections at intervals of 10 to 20 days	5
2	6	SLE virus, routine i.c. passage strain, 10^{-3} s.c. twice 10^{-4} i.c. once	4
3	6	Influenza virus, PR-8, 1/50 i.p. twice	1
4	6	Pertussis vaccine: 5,000 million cells/cc, single injection	1
5	3	Cortisone, 2 mg per mouse, day 0 and day 1	1
6	3	Insulin, 1 unit PZI per mouse, day 0, 1, 2	1

subcutaneously, 9 days apart; virus was recovered from brain (1:30 emulsion) and from pooled spleen, kidney, and liver (1:30 emulsion) on the 5th day after the 2nd injection, but not on the 6th to 9th days, and not from blood at any time. Another group, similarly inoculated, but with a 13-day interval between injections, yielded demonstrable virus in brain emulsion on the 16th day after the second inoculation, with negative tests on the 15th, and on the 17th to 20th days. A 3rd group, which received infected brain subcutaneously, followed by an intracerebral injection (infected brain 10^{-3}) on the 13th day, yielded no demonstrable virus in brains examined from the 15th to the 20th days after the 2nd injection. Other tissues from these last 2 groups were not tested.

The work of earlier investigators, and observations presented here, indicate that the presence of LCM in vital organs of the mouse, including the brain, does not per se initiate significant illness. But virus introduced in a manner —or at a time in the mouse's life—different from that which operates under natural circumstances, may kill the mouse or may itself be eventually suppressed. Suppression of demonstrable virus by the mouse occurred in these studies, and in those described by Traub,[15] much earlier in those artificially induced infections which were of a mild or inapparent nature, e.g., those caused by subcutaneous injection of brain emulsion (table 3). This is in contrast to the situation in the natural type of infection, acquired in utero, where the mild or inapparent infection is not suppressed by the mouse, but survives apparently as long as the host. During these studies, many attempts were made to induce suppression of the infection acquired in utero. Some mice were given homologous virus intracerebrally as many as 5 times; others were given SLE virus subcutaneously, followed by the same virus intracerebrally; still others were given influenza virus (PR-8) intraperitoneally. None of these mice suppressed their original LCM infections (table 4).

Viremia has been recognized as a prominent feature of LCM infection in mice since the earliest studies.[2,13,14] A particularly strong attachment of virus to mouse blood cells was described by Schwartzmann.[16] In the studies reported here, viremia was found in mice inoculated by all three routes (i.c., i.p., s.c.). In a group inoculated subcutaneously with infected blood, virus was recovered from blood cells suspended in saline and from plasma, each at several dilutions up to one-fourth original volume. Though titrations were not made, the number and timing of deaths among test mice suggested that virus was present in blood cells and plasma in about equal quantities. Cells still yielded virus after 2 saline washings.

16. Schwartzmann, G. 1944, J. Immunol. **48**: 111–127.

Alterations in incubation period

For convenience of discussion, the term "incubation period" is used in this section to designate the time required for infection to produce death. For a given dose of LCM and a particular route of injection this is a rather constant period. In 500 mice given brain emulsion 10^{-3} intracerebrally, the 6th and 7th days accounted for 408 deaths; only 3 mice died on the 5th day, and only 10 after the 10th. In 500 mice inoculated intraperitoneally with emulsion of pooled spleen, kidney and liver 10^{-3}, there were 308 deaths, of which 234 occurred on the 6th, 7th or 8th days; only 3 occurred on the 5th, and 36 after the 10th days.

During these studies, the incubation period following intracerebral inoculation has been shortened experimentally. Mice were given infected brain emulsion, 10^{-2} subcutaneously; then on the 3rd day they were injected intracerebrally with infected brain emulsion, 10^{-3} or 10^{-4}. Four experiments of this kind are summarized in table 5 (exps. 1 to 4): they show that incubation periods in specifically prepared mice were shorter than in controls (prepared by subcutaneous injection of emulsion of uninfected brain). Specifically prepared mice began to die on the 4th day, controls on the 6th; mean day of death was from 2.1 to 2.6 days shorter in the specifically prepared groups.

This acceleration of fatalities was accompanied by a sparing effect: though mice which died did so sooner in the specifically prepared series, fewer mice died than in the controls. In the 4 experiments, there were 29 survivals among 64 mice specifically prepared; none of 67 controls survived. Immunity, or something producing a similar effect, developed rapidly, but mice in these groups were subjected to the accompanying alternative of accelerated fatality.

When intracerebral inoculation was given less than 3 days after subcutaneous preparation, no definite effect was observed; as the interval between injections exceeded 3 days, the accelerating effect gave way to the sparing influence (Traub reported no effect from intraperitoneal injections given on the same day as intracerebral challenge; some mice challenged on the 5th day survived; on the 8th day all were immune.[14] He also referred to an "accelerated but non-fatal reaction" to intracerebral challenge on the part of

TABLE 5.—*Alterations in incubation periods in mice given multiple injections of LCM.*

Exp. no.	First injection, dilution expressed logarithmically	Later injections, dilutions expressed logarithmically	Deaths on indicated day after first i.c. inoculation										Mean	Survived
			3	4	5	6	7	8	9	10	11	12–14		
1	LCM (brain)—2 s.c.	LCM (brain)—3 i.c., 3rd day		3		1		1					5.1	7
	Normal brain)—2 s.c.	LCM (brain)—3 i.c., 3rd day				1	7	7					7.4	0
2	LCM (brain)—2 s.c.	LCM (brain)—4 i.c., 3rd day		2	2	3					1		5.8	7
	Normal brain—2 s.c.	LCM (brain)—4 i.c., 3rd day					4	8	1			1	8.0	0
3	LCM (brain)—2 s.c.	LCM (brain)—4 i.c., 3rd day		5	3	0							4.4	2
	Normal brain—2 s.c.	LCM (brain)—4 i.c., 3rd day				1	8	1					7.0	0
4	LCM (brain)—2 s.c.	LCM (brain)—4 i.c., 3rd day		2	8	1							4.9	13
	Normal brain—2 s.c.	LCM (brain)—4 i.c., 3rd day				6	15	5	1				7.0	0
5	LCM (brain)—4 i.c.	LCM (brain)—2 i.p., 6 hr intervals, 16 times	1	7			1	1					4.6	0
	LCM (brain)—4 i.c.	Normal brain—2 i.p., 6 hr intervals, 16 times					1	4	3	2			8.6	0
6	LCM (liver, kidney, spleen)—2 i.p.	LCM (brain)—2 i.p., 6 hr intervals, 16 times	2	5		1	2						4.6	0
	LCM (liver, kidney, spleen)—2 i.p.	Normal brain—2 i.p., 6 hr intervals, 16 times					3	3	2			2	8.9	0

TABLE 6.—*Patterns of LCM development (in vivo) in brains of nonimmune mice, as indicated by numbers and timing of deaths in mice inoculated with brain emulsions prepared at various intervals after intracerebral injection of virus.**

Brain removed and tested at indicated interval after i.c. inoculation	Exp. 1											Exp. 2										
	Mice dying on indicated postinoculation day									Survived	CIVA†	Mice dying on indicated postinoculation day									Survived	CIVA†
	6	7	8	9	10	11	12	13	14			6	7	8	9	10	11	12	13	14		
Immediately		1	3	3						3	34		4	5							0	53
1 hour					1					9	3		1	2	2	1					4	28
2 hours		1	1	1			1			6	17				1	1					8	7
3 hours				2		1				7	11				3		1		1		5	17
4 hours				1	1					8	7					1		1		1	7	6
5 hours					1					9	3			1	1		2				6	15
6 days								1		9	1			1							9	5
1 day	9	1								0	63	1	6	3							0	55
2 days	7	3								0	62	1	7	2							0	56
3 days	2	4	1	1	2					0	52		6	3	1						0	54

* Dilutions and other details explained in text.
† Comparative index of virus activity; explained in text.

mice which retained only "partial immunity" as a result of initial non-fatal infection weeks or months previously).[15]

The incubation period was shortened in another manner during these studies. In one experiment, mice were given infected brain 10^{-4} intracerebrally, followed by infected brain 10^{-2} intraperitoneally at 6-hour intervals for 16 injections; controls received the same intracerebral preparation, but uninfected brain intraperitoneally. The superinfected mice began dying on the 3rd day, the mean day of death being 4.6; controls died first on the 7th day, with mean day of death 8.6 (table 5, exp. 5). In another experiment, the initial injection was made with emulsion of pooled spleen, kidney and liver 10^{-2} intraperitoneally; subsequent inoculations were exactly as in the 1st experiment. Superinfected mice died first on the 3rd day, with mean day of death 4.6; controls began dying on the 7th day, with mean day of death 8.9 (table 5, exp. 6). No mice survived in any of these tests.

Virus development in brains of nonimmune and immune mice

The survival and development of LCM in mouse brains was studied by injecting brain emulsion intracerebrally, then removing whole brains at intervals thereafter, and assaying their virus content by inoculation intracerebrally into fresh mice.

Experiments such as those summarized in table 6 show that virus was readily recoverable from brains immediately after injection, but from the 1st to the 6th hours afterward it was demonstrable to only a limited degree. Either it largely disappeared from the site of injection within a few minutes, or it remained in some form not detectable by the test employed (a circumstance in keeping with that described for herpes virus,[17] the virus of Western equine encephalomyelitis,[18] the bacterial viruses,[19] and others). No tests were made after the 6th until the 24th hour, and from then on virus was readily demonstrable at daily intervals.

In another set of experiments, brains were removed from mice immediately after inoculation, then stored whole in saline at 36 C until removed from virus assay. (In later experiments, undiluted normal mouse serum was used instead

17. Burnet, F. M. and Lush, D. 1939, J. Path. Bact. **49**: 241–259.
18. Schlesinger, R. W. 1949, J. Exper. Med. **89**: 491–505; 507–527.
19. Doermann, A. H. 1948, Yearbook Carnegie Inst. **47**: 176–182.

TABLE 7.—*Patterns of LCM survival (in vitro) in brains of nonimmune mice; all brains removed immediately after inoculation and kept whole (in physiological saline) at 36 C until assayed for virus. Numbers and timing of deaths in mice inoculated with brain emulsions for virus assay.**

Brain tested for virus at indicated interval after i.c. inoculation	Experiment 1										Experiment 2									
	Mice dying on indicated postinoculation day								Survived	CIVA†	Mice dying on indicated postinoculation day								Survived	CIVA†
	7	8	9	10	11	12	13	14			7	8	9	10	11	12	13	14		
Immediately	4	3							0	54	6	3	1						0	54
1 hour	1	6	2	1					0	48	7	1	2						0	54
2 hours	5	2							1	48	4	5							1	48
3 hours	4	5		1					0	51	2	3	2					1	2	36
4 hours	1	7	1						0	50		2	1						4	20
5 hours	2	4	1						0	50			2						8	8
6 hours	1	7	2						0	49	2	2	3	2					1	41
7 days	4	3							0	54		6	1	1	1				1	40
1 day	1	1	4						0	40	1	2	2	1	1	1	1		1	34

* Dilutions and other details explained in text.
† Comparative index of virus activity, explained in text.

of saline.) When kept thus in vitro, virus did not show the lag phase that was observed in vivo (table 7). The pattern of survival suggested a relatively constant quantity during the first 7 hours, and no demonstrable increase on the first postinoculation day.

Serial titrations over a 6-day period, comparing virus survival and development in vivo with that in vitro, indicate steady decline of virus in vitro, contrasting with the high titres developing in vivo (table 8).

The pattern of LCM development in brains of immunized mice was studied by the methods which provided the data for tables 6 and 7. Mice were immunized in two ways: (a) Infected brain emulsion 1:50 was given subcutaneously, and repeated after 2 weeks; after another 2 weeks these mice were employed for virus survival tests and designated "subcutaneously hyperimmunized." (b) The subcutaneous injection was followed in 2 weeks by an intracerebral one (infected brain, 10^{-3}); these mice were ready for use 2 weeks later, and were designated "intracerebrally hyperimmunized."

Control groups were treated as in (b), except that both inoculations were made with uninfected mouse brains.

To compare virus development patterns in mice of these groups by studying data presented in the manner of tables 6 and 7 is too cumbersome. Serial titrations were not made in assaying brains for virus content, so end point comparison is not possible with the data available. In order to provide a simple method of comparing patterns of virus development in these groups of mice, a scheme of indicating relative quantities of active virus, based upon numbers and timing of deaths in test mice, is here employed. (A method of expressing activity of an infectious agent based upon timing of deaths in inoculated animals was described by Golub,[20] who reviewed previous meth-

20. Golub, O. J. 1948, J. Immunol. 59: 71–82.

TABLE 8.—*Titrations of LCM-infected mouse brains, to compare survival and development of virus in vivo and in vitro after i.c. inoculation (brain emulsion, 10^{-3}).*

Postinoculation day	Brains removed from mice on days indicated and assayed by inoculation i.c. into mice at dilutions shown. Numbers indicate assay mice dying per 5 injected (dilutions expressed logarithmically)							Brains removed from in vitro storage (36 C) on days indicated and assayed in same manner as those taken directly from mice				
	2	2.5	3	3.5	4	4.5	5	2	2.5	3	3.5	4
1			5	5	4	3	1					
2	5	5	5	5				5	5	5	5	
3	5	5	5	5				4	5	3	0	
4	5			5	5	5		4	0	3	0	
5			5	5	5	5	5	1	0	0	0	
6			5	5	5	5	5	3	3	2	0	

TABLE 9.—*Patterns of LCM survival and development in mouse brains as shown by recovery of virus at intervals after i.c. inoculation with infected brain emulsion, 10^{-2}. Relative quantities of demonstrable virus expressed as comparative indices of virus activity (explained in text).*

Postinoculation interval	Brains removed from mice at intervals shown; tested for virus by i.c. inoculation into fresh mice (1/30 emulsion). CIVA shown			Brains removed from mice immediately after inoculation; kept in vitro* until tested; virus tests as for those remaining in vivo. CIVA shown		
	Nonimmune mice	Subcutaneously hyperimmune mice	Intercerebrally hyperimmune mice	Nonimmune mice	Subcutaneously hyperimmune mice	Intracerebrally hyperimmune mice
Immediately	9	9	4	12	19	9
1 hour	0	0	0	11	18	11
2 hours	5	0	0	12	9	24
3 hours	0	0	0	4	14	8
4 hours	0	0	5	15	11	9
5 hours	0	0	0	3	3	12
6 hours	0	0	0	12	3	0
1 day	56	54	0	0	6	0
2 days	57	58	4	9	10	0
3 days	58	59	0	0	3	0
4 days	61	21	0	0	0	0
5 days	59	43	0	0	0	0

* Brains kept whole in full strength normal mouse serum at 36 C.

ods developed on similar principles.)

Indices of comparison are calculated as follows:

(a) number of mice in experiment
(b) duration of experiment (days)
(c) maximum possible survival (mouse days): a×b
(d) number of mice alive at end of experiment (survivors)
(e) number of survivors × duration of experiment: d×b
(f) total number of days survived by mice which died; e.g., if 4 mice were found dead on the 6th day, and 3 on the 7th, then total survival of these 7 mice is 4×5 plus 3×6
(g) total actual survival of all mice in experiment (mouse days): e+f
(h) survival deficit, i.e., difference between maximum possible survival (c) and total actual survival (g): c−g
(i) Percent survival deficit: survival deficit (h)/maximum possible survival (c)

For convenient usage, the quantity (i) above, representing survival deficit expressed as percent, is designated "comparative index of virus activity (CIVA)." The indices are included in tables 6 and 7, to show how they relate to actual protocols. In table 9, they are used without presentation of the protocols from which they were calculated.

The experiments summarized in table 9 show that LCM development in mouse brain during the first 6 hours after intracerebral injection follows the same pattern in all 3 categories of mice: nonimmune, subcutaneously hyperimmunized, and intracerebrally hyperimmunized. From the 1st to the 5th days the pattern of development is similar in the nonimmune and the subcutaneously hyperimmune groups. In the latter, the development of considerable quantities of virus in the brain has no apparent effect on the mice, which are uniformly immune in terms of survival. In other tests, not tabulated here, virus has been demonstrated in brains of subcutaneously hyperimmune mice on the 7th and 8th, but not on the 20th days.

In mice of the intracerebrally hyperimmunized series, there was a striking difference in the pattern of LCM development from the first day on: almost no virus was demonstrable by the methods used.

Mice hyperimmunized subcutaneously and intracerebrally, as described, were given a pseudo-challenge of normal mouse brain intracerebrally to determine whether the results obtained in the virus development studies were in fact attributable to the challenge virus or

might have been complicated by persistence of virus from the immunizing injections. These pseudochallenges resulted in no recoveries of demonstrable virus from the hyperimmunized mice.

These patterns of virus survival and development were observed in mice tested about 2 weeks after the hyperimmunizing injection. In another experiment, not tabulated, such patterns were studied in mice inoculated 43 days after hyperimmunization. Virus tended to disappear a little more slowly from the intracerebrally hyperimmunized mice, being equivalent to that in the other 2 groups on the first postinoculation day, but from the 2nd to 4th days quantities demonstrable were minimal, and none was detected on the 5th day. The subcutaneously hyperimmunized and the nonimmune mice showed the same pattern for the first 3 days, but the former yielded less virus on the 4th, and only a trace on the 5th day.

These experiments, along with those described in an earlier section, indicate that mouse survival is compatible with significant quantities of virus in the brain in artificially immunized mice, just as it is in those harboring the natural type of infection. They also show that early complete suppression (for practical purposes) of demonstrable virus in the brain can be induced. The determining factor is directly related in some manner to the site of the hyperimmunizing injection, rather than to the quantity of virus injected, for intracerebrally hyperimmune mice received only 0.05 cc of 10^{-3} brain emulsion at the 2nd inoculation, compared with 0.25 cc of 1:50 emulsion given to those hyperimmunized subcutaneously.

Another set of experiments shows that the pattern of virus survival in mouse brains kept in vitro is the same in all 3 categories of mice (table 9), and corresponds to that already described for nonimmune mice.

Similar patterns of virus development in normal mouse brains have been reported with SLE, which is not known to be a natural parasite of mice.[21] During the present studies, this virus was found to develop in a manner parallel to that of LCM in mice of different immune categories, both in vivo and in vitro (data not tabulated).

Neutralizing antibodies

Mice do not develop antibodies capable of neutralizing LCM in vitro,[14] although other animals do.[4] During the present studies, unsuccessful attempts were made to stimulate production of neutralizing antibodies in mice infected in utero, and in those hyperimmunized artificially. Methods used included repeated injections with homologous virus with SLE, and with influenza virus. No mice developed neutralizing antibodies against LCM detectable by the tests employed (table 10), though antibodies were developed against SLE and influenza viruses (table 11).

Comparison of response of mice to LCM with that generated by SLE shows a dissimilar relationship between immunity to challenge and presence of neutralizing antibodies. A single subcutaneous injection of LCM (in these studies dilutions as high as 10^{-7} were completely effective) stimulates in mice a solid immunity against intracerebral challenge (even in concentrated suspensions of 10^{-2}), but numerous injections of homologous virus produce no serum antibodies capable of neutralizing the virus in vitro (table 10). Subcutaneous inoculation of SLE produces a somewhat less effective immunity to intracerebral challenge than that obtained with LCM, yet it stimulates production of neutralizing antibodies (table 12).

DISCUSSION AND SUMMARY

The natural association of lympho-

21. Webster, L. T. and Clow, A. D. 1936, J. Exper. Med. **63**: 433–448

TABLE 10.—*Failure of mice hyperimmunized against LCM to develop serum antibodies capable of neutralizing virus in vitro (all tests with pooled serums).*

Serum no.	Preparation of mice with LCM passage virus of i.c. strain	Neutralization tests: mice surviving of 5 inoculated at indicated logarithmic dilutions of virus	
		4	5
1a	1 s.c., 2 i.c. injections	0	0
1b	Controls: normal mouse brain injections	1	1
2a	1 s.c., 1 i.c. injection	1	0
2b	Controls: normal mouse brain injections	0	0
3a	1 s.c., 1 i.c. injection	0	1
3b	1 s.c., 2 i.c. injections	0	0
3c	Controls: non-injected normal mice	0	0
4a	Controls for entire above series: Rat: 2 s.c. and 1 i.c. inj. LCM	1	5
4b	Controls for entire above series: Rat: 2 s.c. and 1 i.c. inj. NMB	0	1

cytic choriomeningitis (LCM) virus and the mouse provides for mutual survival: neither parasite nor host suppresses the other to a significant degree, though each is inherently capable of doing so. These inherent capabilities become operative under artificial conditions of transmission.

Several factors influence the development of virus-mouse relationships. One is age of the mouse at time of infection. Mice infected in utero develop the natural type of mutually tolerant relationship with the virus: attempts to upset this relationship were uniformly unsuccessful in these studies. Those infected after reaching maturity enter a relationship that is terminated sooner or later by death of either parasite or host. Milder infections artificially induced die out sooner than severe ones; mice rendered immune by a mild infection readily suppress a subsequent infection with homologous virus.

Another influencing factor is site of injection. In general, subcutaneous inoculation produces mild or inapparent infection. Intraperitoneal and intravenous injections cause severe illness, with many deaths and chronic infections. The mouse tolerates much larger quantities of virus by either subcutaneous, intraperitoneal or intravenous injection than it can withstand intracerebrally.

Additional factors which may influence host-parasite relations probably include passage history of the virus, direct trauma to the brain of the mouse, and sometimes quantity of virus injected.

The presence of virus in vital organs of the mouse, even in relatively large quantities, does not per se cause significant illness.

Incubation period is relatively constant for a given route of inoculation and dose of virus. This period may be altered by several methods of superinfection. In one instance, where sub-

TABLE 11.—*Successful development of neutralizing antibodies against SLE and influenza viruses by mice which failed to develop antibodies capable of neutralizing LCM. Tests done with same pooled serums used for LCM tests.*

Serum	Preparation of mice with SLE or PR-8 after hyperimmunization with LCM	Neutralizing tests: mice surviving of 5 inoculated at indicated dilution of virus		
		SLE		PR-8
		10^{-5}	10^{-6}	1/5000
1	SLE: 3 s.c., 1 i.c. injection	4	4	
2	SLE: 2 s.c. injections	2	2	
3	SLE: 2 s.c., 1 i.c. injection*		3	
4	PR-8: 2 i.p. injections			5
5	PR-8: 3 i.p. injections*			5
6	Controls: noninjected normal mice	0	1	0

* Mice infected in utero with LCM.

TABLE 12.—*Immunity to SLE in mice following subcutaneous injection of potent virus.*

Preparation of mice: this is the same group of mice tested after each injection of SLE	Fatality of SLE infections caused by preparatory injections		Serum neutralization*	
	Mice injected	Deaths	Number of mice surviving of 5 injected with mixtures of serum and indicated logarithmic dilutions of virus	
			5	6
SLE 10^{-3} s.c.; 1 injection	60	6	2	4
SLE 10^{-3} s.c.; 2 injections, 2 weeks apart	54	1	2	4
SLE 10^{-3} s.c.; 2 injections, 2 weeks apart; 10^{-3} i.c. 2 weeks later	41	5	1	5
Controls: no preparation			0	2

* Serums taken 2 weeks after last SLE injection.

cutaneous injection is followed by intracerebral inoculation 3 days later, the development and fatal outcome of the intracerebral infection is accelerated in some mice, but others in the same group are spared. A mouse specifically prepared in this fashion prior to a normally fatal intracerebral inoculation faces two alternatives: either it dies more quickly as a result of inoculation or it completely escapes visible illness.

Shortening of the incubation period can also be induced by repeated injections with homologous virus at frequent intervals.

The pattern of LCM development in mouse brains is this: within the 1st hour after intracerebral inoculation, demonstrable virus diminishes greatly in quantity or disappears; by the end of the first 6 hours there has been no detectable change in this situation. However, by the 24th hour after inoculation, demonstrable virus has increased greatly in the living mouse. In brains removed immediately after inoculation and kept in vitro there is no lag period, but a gradual decline in quantity of virus takes place over several days.

Subcutaneously induced infections hyperimmunize mice against intracerebral challenge, but challenge virus is recoverable from the brain in significant quantity for more than a week after injection. The developmental pattern is indistinguishable by the tests employed from that in the nonimmune mouse, but the hyperimmunized mouse survives (in spite of the virus in its brain), while the nonimmune dies.

Mice immunized by a single subcutaneous injection, and later hyperimmunized by intracerebral inoculation, sustain a different reaction to later intracerebral challenge. In these, there is little or no recovery of demonstrable virus from the brain after the initial lag period, and virus is seldom encountered, except in minimal quantities, beyond the 1st postinoculation hour. Mice hyperimmunized by 2 subcutaneous injections receive much greater quantities of virus than those hyperimmunized intracerebrally; therefore, the route of injection must have a determining influence on reaction to subsequent challenge.

In mouse brains removed immediately after intracerebral inoculation and kept in vitro, there is no difference in the pattern of virus survival among the three categories of mice.

Mice do not develop serum antibodies capable of neutralizing LCM in vitro, even though solid immunity to challenge is readily producible. This situation is different from that in SLE infection, where mice develop neutralizing antibodies, but resistance to challenge is less effective than in LCM infection.*

* I wish to acknowledge the valuable assistance rendered to this study by my helper, Roskey I. Jennings.

THE SEPARATION AND ANALYSIS OF LABELLED ANTIBODIES

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Antibody proteins with a variety of specificities have been trace-labelled with I^{131} without loss of immunologic activity.[1–4] Despite this fact, labelled antibodies have not found the wide experimental usage that might be expected, largely because of the difficulty in separating the labelled antibodies from the large background of nonspecific, protein-bound radioactivity. Most of the work with labelled antibodies reported to date has required a separation of the specific antibody prior to the radioactivity analysis of each sample, either by precipitation with a soluble antigen,[2–8] or by absorption by an insoluble antigen.[1,9–11] An initial concentration of the labelled antibody has been obtained by Pressman in an effort to increase the significance of radioactivity determinations.[12,13] His methods, however, are applicable to insoluble antigens only and involve a degree of heat or alkali which may be injurious to the immunologic activity of the antibody. The results which he reported do not permit an estimation of the degree of antibody purity obtained, nor an analysis of other characteristics of the labelled antibody.

Theoretically, labelled antibody solutions with high isotopic specificity (high percentage of antibody bound radioactivity) could be produced in either of these two ways: (1) By labelling previously purified antibody solutions or (2) by labelling impure antibody solutions and separating the labelled antibody from the other labelled substances.

The chemical purification of antibody, a prerequisite of the first method, has been accomplished by both specific and nonspecific methods. For details the reader is referred to recent reviews.[14,15] In general, nonspecific methods have resulted in a degree of purity inadequate for isotope experiments. Antibody of high degree of purity has been obtained for a fairly wide range of antigens by various methods of specific purification in which the antigen-antibody union was

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1. Butement, F. D. S. 1948, J. Chem. Soc. suppl. **2**: 408.
2. Masouredis, S. P., Melcher, L. R. and Koblick, D. C. 1951, J. Immunol. **66**: 297–302.
3. Cohen, S. 1951, J. Immunol. **67**: 339–346.
4. Francis, G. E., Mulligan, W. and Wormall, A. 1951, Biochem. J. **48**: 36–37.
5. Banks, T. E., Francis, G. E., Mulligan, W. and Wormall, A. 1951, Biochem. J. **48**: 180–187.
6. Melcher, L. R. and Masouredis, S. P. 1951, J. Immunol. **67**: 393–401.
7. Green, H. and Anker, H. S. Biochemica et Biophysica Acta, In press.
8. Maurer, P. H. and Talmage, D. W. 1953, J. Immunol. **70**: 435–440.
9. Pressman, D. and Keighley, G. 1948, J. Immunol. **59**: 141–146.
10. Eisen, H. N., Sherman, B. and Pressman, D. 1950, J. Immunol. **65**: 543–558.
11. Pressman, D. and Eisen, H. N. 1950, J. Immunol., **64**: 273–279.
12. Pressman, D., Sherman, B. and Korngold, L. 1951, J. Immunol. **67**: 493–500.
13. Korngold, L. and Pressman, D. 1953, J. Immunol. **71**: 1–5.
14. Campbell, D. and Lanni, E. 1951, Amino Acids and Proteins, Chap. XI, Springfield, Illinois, C. C Thomas.
15. Kabat, E. A. and Mayer, M. M. 1948, Experimental Immuno-Chemistry; Chap. 43, Springfield, Illinois, C. C Thomas.

dissociated at an acid[16,17] or alkaline[18] pH. Significant dissociation at a neutral pH has been possible only with special systems in which an antigen competitor was available.[19,20] Attempts to label with I^{131} the antibody dissociated at an acid or alkaline pH have been unsuccessful in this laboratory because of the instability and high dilution of the antibody so produced.

The preparation of labelled antibody solutions described in the present report makes use of the second of the two possible methods listed above. Labelling the antibody before its separation has the disadvantage of wasting radioactivity, but was found to provide two important advantages over the previous attempts to label a chemically pure antibody:

(1) The separation of labelled antibody is simpler and requires less drastic changes in temperature or pH than the chemical purification of an unlabelled antibody. This is due to the fact that unlabelled carrier proteins can be used to aid in the separation of labelled antibody. The method described in this report, using unlabelled antibody to compete with labelled antibody, is somewhat analogous to previously reported methods of purifying antibody, using an antigen competitor.[19,20]

(2) The high concentration of protein present in the unpurified globulin fraction of an antiserum simplifies the iodination of the antibody by permitting the use of a relatively large amount of carrier iodide without damage to the immunologic activity of the antibody.

The techniques of separation and analysis described in the present report have been worked out with rabbit antiserum to human γ globulin. They should be broadly applicable, however, to any antigen-antibody system in which the antigen (1) is insoluble to begin with, (2) can be made insoluble by attaching it to cellulose or some other insoluble compound, or (3) can be precipitated with specific antibody. The methods reported here have been successfully applied in this laboratory to two other systems: (1) antisheep cell hemolysin and (2) anti-tissue antibodies. Results from these systems will be reported separately.

PREPARATION OF ANTIGENS

Lederle's immune serum globulin (human γ globulin, or HGG) #2167–98 was used throughout.

An insoluble antigen was made by binding the HGG to Solka Floc† BW 200, according to the method described by Campbell, Leuscher and Lerman.[16] The globulin was added to the *p*-aminobenzylcellulose at a ratio of 1 to 7 by dry weight. No attempt was made to analyze the final HGG cellulose product for antigen content. However, absorption experiments described below indicate that the cellulose antigen was capable of absorbing at least 1/30th of its weight of antibody.

To provide a control absorbent, Cutter's human serum albumin (HSA) was combined with the cellulose in a similar manner to the HGG.

PREPARATION AND FRACTIONATION OF ANTISERUM

Rabbits were injected at approximately monthly intervals with a water-in-oil emulsion[21] containing human γ

16. Campbell, D., Luescher, E. and Lerman, L. S. 1951, Proc. Natl. Acad. Sc. **37**: 575.
17. Eisen, H. N. and Karush, F. 1949, J. Am. Chem. Soc. **71**: 363.
18. Sternberger, L. A. and Pressman, D. 1950, J. Immunol. **65**: 65–73.
19. Isliker, H. C. Ann. N. Y. Acad. Sc. In press.
20. Karush, F. 1953; Federation Proc. **12**: 448.

† Kindly supplied by Brown Co., Chicago, Ill.

21. Talmage, D. W. and Maurer, P. H. 1953, J. Infect. Dis. **92**: 288–300.

globulin. On each occasion each rabbit was given a total of 50 mg HGG in 4 ml of emulsion distributed over four sites, two intramuscular, and two subcutaneous.

Rabbits were bled by intracardiac puncture 10 days after each injection except the first, and preliminary antibody determinations done on each serum by the labelled antigen precipitation method.[21] The number of P_{80} units of antigen precipitation was multiplied by 3.5 to give the approximate antibody N content. Serums of approximately the same antibody content were pooled.

The globulin fraction of the most concentrated antiserum available (500 μg antibody N/ml) was separated by precipitation with ammonium sulfate at 50% saturation. The precipitate was dissolved in one half the original volume of water, then dialyzed against running tap water overnight, and for 3 days against daily changes of 0.85% NaCl in the cold. The solution was brought to a pH of 7.2 to 7.6 with 0.05 *M* Na_2HPO_4, diluted to contain 20 mg protein /ml and stored in ampules containing 0.5 ml each in the deep freeze. Merthiolate was not added, since this was found to reduce the iodine uptake by protein, particularly at a neutral pH.

LABELLING WITH I^{131}

Because of the short half-life of I^{131} it was necessary to make preparations of labelled antibody at frequent intervals. For this reason the following simple and rapid procedure was worked out to permit the iodination of small quantities of serum globulin (as little as 10 mg protein).

Preparation A.—To 0.5 ml of globulin solution containing 10 mg protein was added 0.5 ml of a 0.2 *M* carbonate buffer, pH 10. If 10 mg protein and 0.05 ml of 0.01 *N* KI carrier are used as prescribed below, the iodinated protein will contain an average of 3 atoms of iodine per molecule of globulin, assuming the optimal uptake of 35% of the iodine.

Preparation B.—To approximately 1 mc I^{131} in a volume of 0.5† ml or less the following were added in rapid order:

(1) 0.05 ml of a 0.01 normal solution of KI, freshly diluted from stock 1 N solution.

(2) One drop of 0.5 *N* HCl.

(3) One drop of 0.1 *N* $NaNO_2$. A faint orange iodine color should be visible at this point. If the iodine color was not obtained, an applicator stick and nitrazine paper were used to determine if the solution was acid. If the solution was not acid, 0.5 *N* HCl was added dropwise until acid.

(4) The buffered solution of globulin (preparation A above). The iodine color should disappear promptly and a test with nitrazine paper should show a definitely blue color. If not, 0.5 ml of 0.2 *M* carbonate buffer, pH 10, was added.

(5) One drop of 1 *N* KI. Since the solution is already alkaline at this point, the KI added here does not react with the protein but acts as a carrier in the process of separating the nonprotein bound radioactivity.

A major portion of the 65 or more percent of radioactivity which is not protein bound may be removed rapidly by the dropwise addition of the iodinated protein solution to a 15×150 mm column of amberlite 1R-4B. The protein solution was followed with the dropwise addition of at least five 3 ml portions of distilled water and each portion collected in a separate test tube. The tubes were then monitored and the bulk of the

† A larger volume will result in a decreased iodination efficiency unless the amount of carrier KI is correspondingly increased.

radioactivity usually found in two or three tubes. These tubes were pooled and 1 part in 10 added of a solution of 9% NaCl containing 1:1000 merthiolate and buffered with phosphate to a pH of 7.0. Eighty-five to ninety-eight percent of the radioactivity was found to be protein-bound at this point. Complete removal of nonprotein-bound radioactivity is not required, since this is accomplished in the course of the antibody separation process described below.

SEPARATION OF LABELLED ANTIBODIES

Previously reported methods for purifying antibodies[12–20] have contained three general steps in common:

(1) Precipitation of the antibody with a soluble antigen or absorption by an insoluble antigen.

(2) Washing the specific precipitate.

(3) Elution of antibody from the specific precipitate.

The procedures reported below for separating labelled antibodies from nonspecific labelled substances all contain the above three general steps, and differ only in the use of nonradioactive carrier proteins. The general principle of carrier exchange, illustrated in figure 1, has been applied to a soluble as well as an insoluble antigen and to elution at an acid as well as a neutral pH. The procedure of choice will depend on the antigen-antibody system and the purpose for which the labelled antibody is prepared. Several of the possible procedures are described below and the characteristics of the eluates obtained are compared.

A. Absorption by an insoluble antigen

Ten ml of labelled anti-HGG globulin solution (I*anti-HGG containing approximately 100 μg antibody N) and 1 ml normal rabbit serum were mixed first with the nonspecific antigen absorbent (0.2 g HSA cellulose, dry weight) for 10 minutes at room temperature and centrifuged 10 minutes at 2000 r.p.m. The purpose of this step is to remove

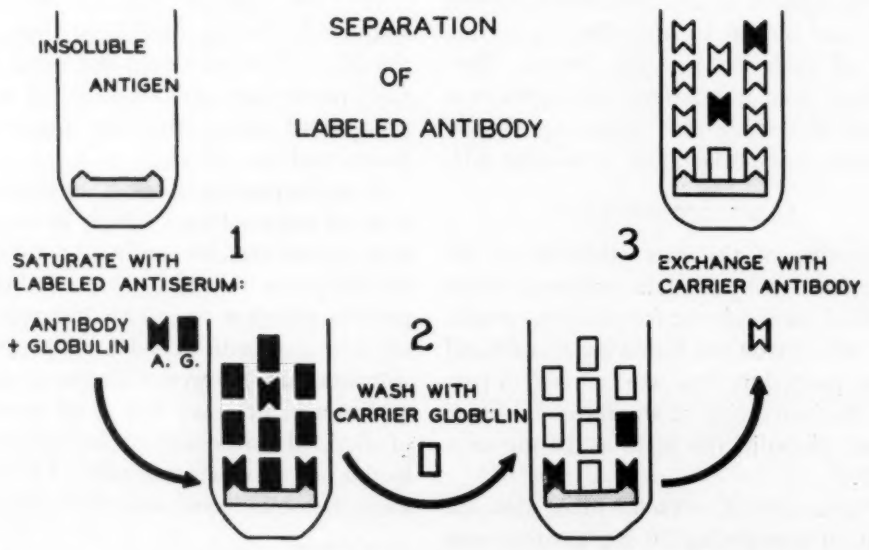

FIG. 1.—A diagrammatic representation of the separation of labelled antibody by exchange with unlabelled carrier antibody.

labelled material easily absorbable nonspecifically.

The supernatant was added to a 50 ml centrifuge tube containing approximately 50 mg HGG cellulose (dry weight estimated to give slight antibody excess). The mixture was kept suspended for 10 minutes at room temperature, centrifuged and decanted.

The cellulose antigen was washed 5 times as follows: 1 ml normal rabbit serum was added and the suspension incubated for 5 minutes at 37 C. It was then cooled in an ice bath and 10 ml cold saline† added. The antigen was suspended and immediately centrifuged. The initial incubation with normal rabbit serum was at 37 C to increase the rate of exchange between labelled and unlabelled globulin nonspecifically adsorbed to the cellulose antigen. Saline was added to increase the efficiency of the washing process but this procedure was done in the cold to counteract with a reduction in temperature the elution of antibody caused by an increase in volume.

1. Elution by exchange at neutral pH.—At the completion of the washing process, 5 ml pooled anti-HGG serum (containing 300 μg antibody N/ml) and 20 ml saline were added to the cellulose-antigen and the mixture kept suspended for 1 hour at 45 C. The suspension was centrifuged and the supernatant (neutral exchange eluate) decanted.

2. Elution at pH 3.2.—Five ml normal rabbit serum and 5 ml saline were added to the sediment remaining from the neutral elution. The pH was brought to 3.2 by the dropwise addition of 0.2 *M* citric acid. The mixture was kept suspended for 15 minutes, centrifuged for 5 minutes, and the supernatant decanted and immediately neutralized to a pH of 7.5 with 0.5 *N* NaOH added dropwise. This is referred to as a citric acid eluate following neutral exchange. An alternative to this elution is direct citric acid elution, omitting the neutral exchange step.

For comparison, the antibody was eluted according to the method of Campbell, Leuscher and Lerman,[16] which uses dilute HCl to a pH of 3.2, and omits the normal rabbit serum.

3. Elution by exchange at pH 3.2.—The acid elution and exchange processes may be combined by substituting 5 ml pooled antiserum for the normal rabbit serum used in the citric acid elution procedure. This is referred to as a citric acid exchange eluate.

B. Precipitation with soluble antigen

To 4.5 ml of I*anti-HGG solution (containing a total of approximately 50 μg antibody N) was added 0.5 ml anti-HGG serum (containing 300 μg antibody N per ml) and 48 μg HGG N. The mixture was incubated for 30 minutes at 37 C and allowed to stand overnight in the refrigerator. The precipitate was then centrifuged and the supernatant decanted. The precipitate was washed 3 times at room temperature with 2 ml saline and 0.5 ml normal rabbit serum.

Elution by exchange at pH 3.2.—The labelled antibody in the precipitate was eluted with 5 ml anti-HGG serum and the dropwise addition of 0.2 *M* citric to a pH of 3.2. After being suspended 15 minutes at room temperature the precipitate was centrifuged off and the pH of the supernatant was brought to 7.5 with 0.5 *M* NaOH added dropwise. The mixture was allowed to stand overnight

† A mixture of 9 parts 0.15 *N* NaCl and 1 part 0.15 *N* KI.

in the refrigerator and the precipitate centrifuged off.

ANALYSIS OF LABELLED ANTIBODY SOLUTIONS

A. Yield

All the fractions obtained from the separation process were analyzed for radioactivity in a well type γ counter. The fraction of the counts in the crude I*anti-HGG globulin which was absorbed or precipitated by antigen approximated 3% in every case. This represents the fraction of the radioactivity in the labelled globulin solution which was attached to antibody molecules.

The efficiency of the elution process was judged by the fraction of the absorbed labelled antibody which could be eluted. The results obtained from the various elution procedures are given in table 1 under the heading of "yield." It is apparent that the highest yields are obtained with the combination of acid elution and carrier exchange.

B. Relative specific activity

The specific activities of the various eluates will depend on (1) the ratio of radioactivity to protein used in iodination, (2) the efficiency of iodination, (3) the ratio of carrier antibody to labelled antibody used in elution, and (4) the percent labelled antibody eluted. Since these factors may vary considerably, the relative specific activity figures quoted in table one are those calculated on the assumption of certain standard conditions. In order of magnitude they approximated experimental results and permit a more valid basis for comparing the various elution procedures.

In eluates obtained with carrier antibody, the antibody concentration is known from the amount of carrier antibody added. The assumption may be made that the antibody concentration is not significantly changed during the exchange procedure.

In eluates obtained with acid alone, the antibody concentration may be too low to be obtained by precipitin determinations. In this case the specific activity of the crude labelled globulin solution may be used, the assumption being made that antibody globulin is labelled to the same degree as the other globulins in the solution.[2,3,8]

The high specific activity of the eluates obtained with acid alone may be valuable in certain in vitro experiments when it is desirable to have high counts in the region of antigen excess.

C. Isotopic specificity of the antibody label

By isotopic specificity of the antibody label is meant the percentage of

TABLE 1.—*A comparison of eluates obtained with soluble and insoluble antigens and at neutral and acid pH.*

Antigen	Method of elution	Average observed yields (percent eluted)	Relative specific activity (counts/μg antibody N)*	Avidity	In vivo stability (See table 4)
HGG-cellulose	Neutral exchange	28	700	Low	Normal
HGG-cellulose	Citric acid following neutral exchange	32	Not determined	Normal	Normal
HGG-cellulose	Citric acid	25	62,500	Not tested	Not tested
HGG-cellulose	Dilute HCl	25	62,500	Not tested	Reduced
HGG-cellulose	Citric acid exchange	67	1,675	Normal	Not tested
Soluble HGG	Citric acid exchange	68	1,700	Normal	Not tested

* The figures quoted in this column are not observed figures, but have been calculated on the basis of a 33 percent uptake of 1 mc I^{131} by 10 mg globulin, the use in the exchange process of an amount of carrier antibody equal to 25 times the labelled antibody absorbed, and a counter giving 300 million counts per mc per minute. In order of magnitude they approximate counts actually obtained.

radioactivity attached to antibody. This percentage is determined by the absorption and precipitation methods to be described (sections C and H). In the crude labelled globulin fraction, approximately 3% of the radioactivity was antibody bound, while after the separation process over 80% of the label was bound to antibody. The percentage values obtained from the absorption and precipitin analyses should be taken as minimum values of isotopic specificity, since the absorption and precipitation of labelled antibody is apparently never complete and varies from preparation to preparation and also with the conditions of the experiment, such as antigen concentration and temperature. Even under optimum conditions of absorption and precipitation, a considerable percentage of the radioactivity in the supernatants may be bound to antibody (items 3 and 4, table 2).

Absorption of labelled antibody by insoluble antigen.—The absorption of a labelled antibody by an insoluble antigen can be measured by mixing the insoluble antigen with the labelled antibody solution, centrifuging off the antigen and counting the two fractions: (1) the absorbent, containing combined labelled antibody and (2) the supernatant, containing uncombined radioactivity. Before calculating the fraction of radioactivity absorbed, it is necessary to correct for the unknown amount of undecanted supernatant present in the absorbent tube. A thorough washing of the absorbent would remove this undecanted supernatant but might also elute some of the absorbed antibody. Consequently, determinations were made of the weight of the water in both tubes and a correction for undecanted supernatant made from these determinations. The details of this correction and a discussion of its accuracy are

TABLE 2.—*Typical analyses illustrating weighed tube method of calculating absorbed fraction.*

Column:			A	B	C	D	E	F	G	H	I	J	K	L	M	N
Obtained from:					A−B		C+D	E/D		G×F	H−G		J−I	H+K	K/L	I/10K
Item	Solution	Antigen	Wet Wt. Abs.	Dry Wt. Abs.	H_2O in abs.	Wt. sup.	Total H_2O	Correction factor	Sup. count	Corrected sup. count	Correction	Abs. count	Corrected Abs. count	Total count	Absorbed fraction	Percent error
1	Eluate-N	HGG-cellulose	152	10	142	1020	1162	1.139	1677	1912	235	4395	4160	6072	0.685	0.6
2	Eluate-N	HGG-cellulose	415	98	317	1023	1340	1.309	906	1187	281	5613	5332	6519	0.818	0.5
3	Sup. abs.	HGG-cellulose	256	14	242	955	1197	1.263	2589	3244	655	2257	1602	4846	0.330	4.1
4	Sup. ppt.	HGG-cellulose	268	14	254	776	1030	1.327	827	1097	270	1372	1102	2199	0.501	2.4
5	Eluate-N	Alb-cellulose	120	8	112	1479	1591	1.076	11894	12800	906	1114	208	13008	0.016	44
6	I*glob.	HGG-cellulose	132	9	123	1482	1605	1.083	25821	27960	2139	2381	242	28202	0.008	88

Percent error (column N) is the percent error induced in the absorbed fraction by a 10 percent error in the supernatant correction (column I). Eluate-N is an exchange eluate obtained at neutral pH. Sup. abs. is the supernatant of an absorption of eluate-N by excess cellulose antigen. Sup. ppt. is the supernatant of a precipitation of eluate-N at equivalence. I*glob. is antibody-free labeled globulin. Alb-cel is human serum albumin cellulose. Conditions of absorption: excess antigen, room temperature, 10 minute reaction time. A more rapid calculation of column M may be made by the formula $M = \frac{DJ - CG}{DJ + DG}$.

found below and in table 2.

Immediately before the addition of labelled antibody, the antigen was washed with both saline and normal rabbit serum. This served two purposes: (1) to remove any soluble antigen which may have been released from the cellulose, and (2) to saturate with unlabelled protein any nonspecific protein binding sites which might be present on the cellulose antigen. After washing, the antigen was transferred to a graduate and saline added to an arbitrary volume. Aliquots estimated to contain 10 mg cellulose antigen (dry weight) were pipetted into weighed tubes. Excess liquid was decanted after centrifugation. One tube was heated at 70 C in order to determine the dry weight; the others were used immediately for the analysis of labelled antibody solutions.

Unless otherwise specified, all absorption studies were made under the following conditions: 0.5 to 1 ml of saline containing the labelled antibody and 0.5 ml of normal rabbit serum were added to the 10 mg antigen in the weighed Wassermann tube. The tubes were shaken and the supernatants decanted into weighed Wassermann tubes. The weight of the contents of both the supernatant (column D, table 2) and absorbent tubes (column A) were obtained by subtraction after reweighing the tubes, and the weight of the liquid in the absorbent tube (column C) obtained by subtracting the known dry weight of the cellulose antigen (column B) in that tube from the total weight of the contents of the tube. If no washings were to be done, the supernatant and the absorbent tubes were counted in a well type γ counter or scintillation counter after adding 1 ml saline to the absorbent to equalize the geometry of the counting. As illustrated in table 2 a correction is made to the supernatant count for the fraction of supernatant remaining after the initial decanting (column F). The error of this correction is estimated to be not greater than 10%. The error in the final absorbent count induced by the error in this correction (column N) will vary, depending on how large the correction is relative to the absorbent count. When the absorbed fraction (fraction of total counts present in the corrected absorbent count) is higher than 0.50, this final error is usually 2% or less. When the absorbed fraction is around 0.20, the final error is around 10%, and with absorbed fractions decreasing below 0.20, the final error increases rapidly and may be over 100%.

Washing.—Since the absorption of antibody by antigen is partially reversible, washing the absorbent will always elute some of the antibody initially absorbed. For this reason washings were kept to the minimum number required to reduce to 2% the error in the absorbed fraction induced by the correction for undecanted supernatant. Beyond this point, additional washing increases rather than decreases the error in the determination. As a general rule, no additional washings were made when the counts in the absorbent were equal to or greater than the counts in the last washing. When working with purified antibody solutions, no washings were performed, whereas with impure labelled globulin fractions one or more washings were necessary, depending on the antibody content of the original antiserum. When washing of the absorbent was done, 0.5 ml of normal rabbit serum was added to the tube containing the absorbent (to which 1 ml of saline had been added prior to counting). The tubes were shaken at room temperature for 10 minutes, and the procedures for centrifugation and counting were repeated as in the previous section.

Controls.—In any experiment involving the absorption of labelled antibody by insoluble antigen, the following two controls were considered necessary:

(*a*) *Antigen control.*—The absorption of antibody-free labelled globulin by the same antigen used in the experiment (item 6, table 2). This control measures nonspecific absorption of labelled globbulin by the antigen in question. Antibody-free labelled globulin was obtained by repeated absorption by the insoluble antigen of the labelled globulin solution.

(*b*) *Antiserum control.*—The absorption of labelled antibody by an unrelated insoluble antigen of comparable physical state (item 5, table 2). This control is necessary to determine the stability of the labelled antibody solution. It is possible that some of the radioactivity of the labelled antibody solution is attached to protein which is easily absorbed on the surface of any insoluble particle. Without this control, such radioactivity might be counted as antibody. In these experiments, cellulose-serum albumin was used as the control antigen.

D. Labelled antibody absorption curve

To equal aliquots of HGG cellulose containing 11 mg dry weight each were added mixtures of labelled and unlabelled antibody containing increasing amounts of total antibody. The final volume was kept constant at 1.5 ml and the total rabbit serum added at 1.0 ml. The antigen was kept suspended on a shaker for 5 hours at room temperature and the absorbed fraction determined according to the method already described. The results are given in figure 2. The antibody absorption curve may be divided into antigen excess and antibody excess zones. In the zone of antigen excess the percent of antibody absorbed is constant, being independent of the ratio between antibody and antigen. In the zone of antibody excess the percent of antibody absorbed decreases

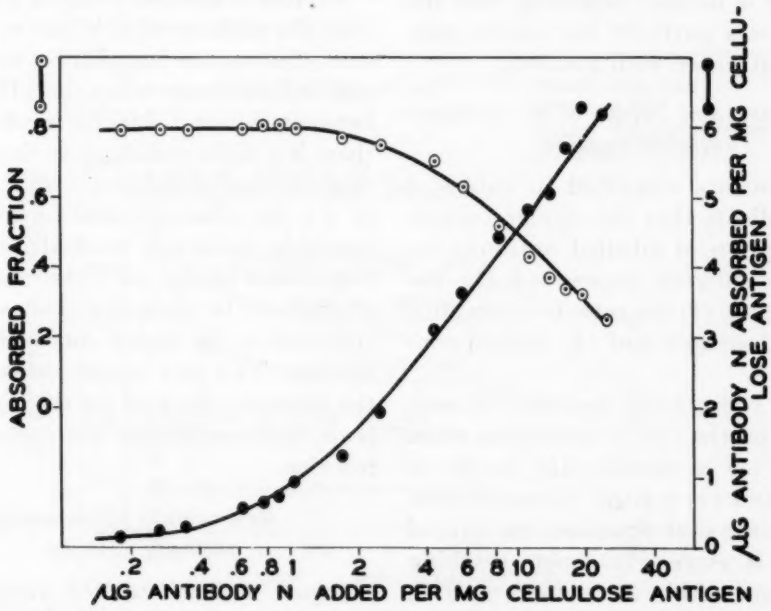

FIG. 2.—The absorption by HGG-cellulose of I*anti-HGG in acid eluate, at room temperature and with 5 hour reaction time.

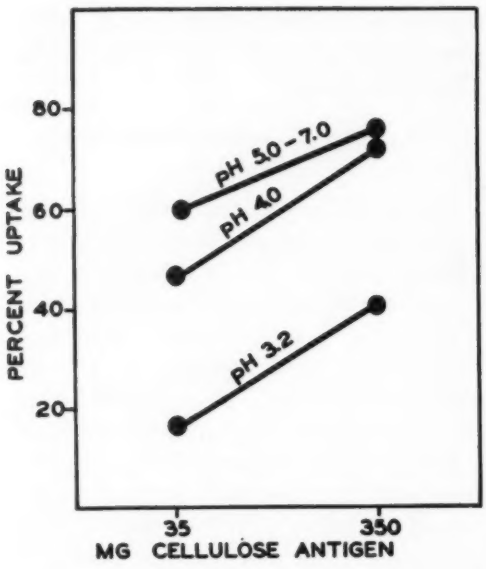

FIG. 3.—The absorption of HGG-cellulose of I* anti-HGG in acid eluate, under varying conditions of pH and antigen concentration. Conditions of analysis: room temperature, antigen excess and 10 minute reaction time.

with increasing ratio of antibody to antigen. The total antibody absorbed continues to rise even while the percent absorbed is falling, indicating that the antigen was partially but never completely saturated with antibody.

E. Velocity and extent of the antigen-antibody reaction

Experiments described in tables 2 and 3 indicate that the rate and extent of absorption of labelled antibody by insoluble antigens depend on (1) the temperature, (2) the ratio between antibody and antigen and (3) antigen concentration.

Table 3 illustrates the effect of temperature on the rate of absorption when there is (1) a considerable excess of antigen and (2) a slight excess of antibody. In the first situation, the rate of reaction is extremely rapid, reaching equilibrium within 20 minutes at 0 C, and 10 minutes at room temperature. When the amount of antibody was adjusted to give a slight excess the rate of the reaction was considerably slower. Equilibrium was reached in 20 minutes at 45 C, 1 hour at room temperature, and was probably not complete in 5 hours at 0 C. The slower velocity of the reaction in the region of antibody excess is probably due to the fact that partial saturation of the antigen results in steric hindrances which slow down the reaction between antigen and antibody.

In the region of considerable antigen excess (table 3) the effect of temperature on the maximum percent of labelled antibody absorbed was not significant (table 3), while the effect of antigen concentration was more pronounced (table 2 and fig. 3). In the region of slight antibody excess, the effect of temperature on maximum absorption is more apparent, the absorption of antibody being more complete at lower temperatures.

F. Effect of pH on the antigen-antibody reaction

Results illustrated in figure 3 indicate that the addition of acid has no significant effect on the reaction between antigen and antibody when the pH is kept between 5.0 and 7.0. At a pH of 4.0 there is a slight reduction in the absorption of labelled antibody, and at a pH of 3.2 the absorption of antibody by insoluble antigen is markedly reduced. Regardless of the pH, the absorption of antibody by antigen was always more complete at the higher concentration of antigen. This fact would indicate that the primary effect of the decreased pH is on the equilibrium constants of the reaction.

G. Reversibility of the antigen-antibody reaction

Figure 4 illustrates the varying degrees of reversibility of the antigen-antibody reaction. Tubes were set up

TABLE 3.—*Velocity of reaction. Percent I^{131} absorbed from neutral exchange eluate by HGG-cellulose.*

	Temperature	1 minute	5 minutes	10 minutes	20 minutes	1 hour	5 hours
Antigen excess	0	47		82	87		
Antigen excess	25	75		86	86		
Slight antibody excess	0	42	51	62	65	79	79
	0	40	51	62	67	76	81
Slight antibody excess	25	50	64	69	73	78	78
	25	48	66	67	72	77	80
Slight antibody excess	45	45	64	64	70	69	66
	45	48	62	63	70	67	67

in the region of slight antibody excess (upper set of curves) using HGG-cellulose and a labelled antibody obtained by elution with citric acid alone. After 1 hour at 0 C, an attempt was made to reverse the antigen-antibody reaction with heat alone, with heat plus an increase in volume, or with heat plus a large excess of carrier antibody. It is apparent that there was some reversal of the antigen-antibody reaction due to the increase in temperature alone, and additional reversal of the reaction when increase in temperature was combined with an increase in volume. The greatest reversal came when carrier antibody was added, but even this was not complete, since the percent of labelled antibody absorbed remained considerably higher than that obtained when the large excess of the carrier and labelled antibody was mixed initially before addition of the antigen (lower curve).

The varying degrees of reversibility of the antigen-antibody reaction may be taken as evidence for the spectrum of antibody avidities referred to by Jerne.[22]

22. Jerne, N. K. 1952, Acta path. et microbiol. Scandinav. suppl. 87.

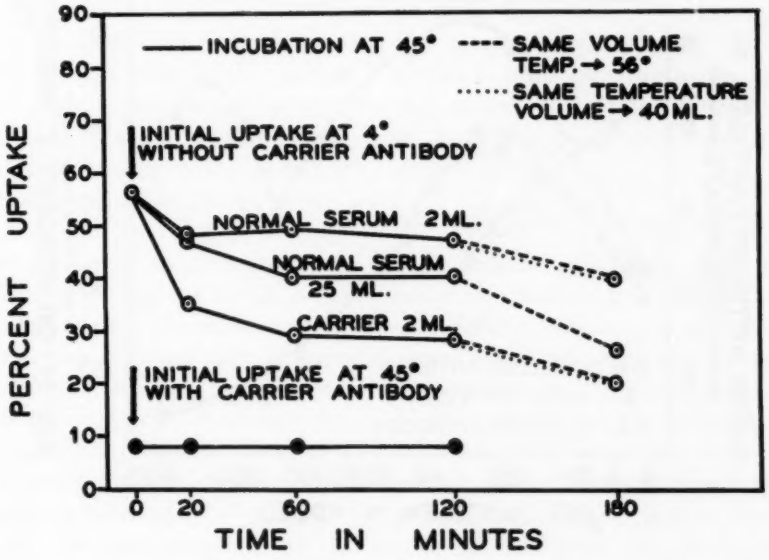

FIG. 4.—The reversibility of the I*anti-HGG and HGG-cellulose reaction under varying conditions of temperature, antigen concentration and carrier antibody. I*anti-HGG is from an acid eluate.

TABLE 4.—*In vivo stability of separated labelled antibody.*

Eluate	Percent of per kg injected dose remaining in 1 g blood on day:								$t_{\frac{1}{2}}$ days 2–7
	0	1	2	3	4	5	6	7	
Citric acid	1.71	0.80	0.60	0.49		0.36	0.35	0.31	5.7
Citric acid	1.46	0.85	0.63	0.52		0.41	0.43	0.35	5.8
HCl	1.50	0.38	0.19	0.18		0.11	0.09	0.07	3.4
Neutral exchange	1.49	0.91	0.65	0.59		0.46	0.38	0.35	5.6
Neutral exchange	1.64	0.74	0.59	0.50		0.39	0.35	0.28	5.2

The acid eluates were obtained at pH 3.2 following an exchange elution at neutral pH.

H. Labelled antibody precipitin curve

Each labelled antibody solution was diluted with carrier antibody and saline to give a final concentration of approximately 100 μg antibody N/ml. To 1 ml aliquots of the mixture of labelled and unlabelled antibodies were added increasing quantities of soluble unlabelled antigen in a 0.5 ml volume. A control tube contained antibody but no antigen. The tubes were capped, mixed and incubated 30 minutes at 37 C and then kept in the refrigerator for 5 to 7 days, being resuspended once each day. At the end of this time, the precipitates were separated by centrifugation and the precipitate fraction determined in a similar manner to that used in the previous sections to determine the absorbed fraction.

The results for three acid eluates and one neutral eluate are presented in figures 5 and 6. A quantitative precipitin nitrogen curve determined with the Folin-Cicolteau phenol reagent is given in figure 5 for comparison. From the total nitrogen curve it is evident that the original antiserum contained two antibodies, the anti-HGG and an antibody to a contaminant in the antigen. Since the second peak is not present in the labelled antibody curve, it is apparent that the antibody to the contaminant has been eliminated in the separa-

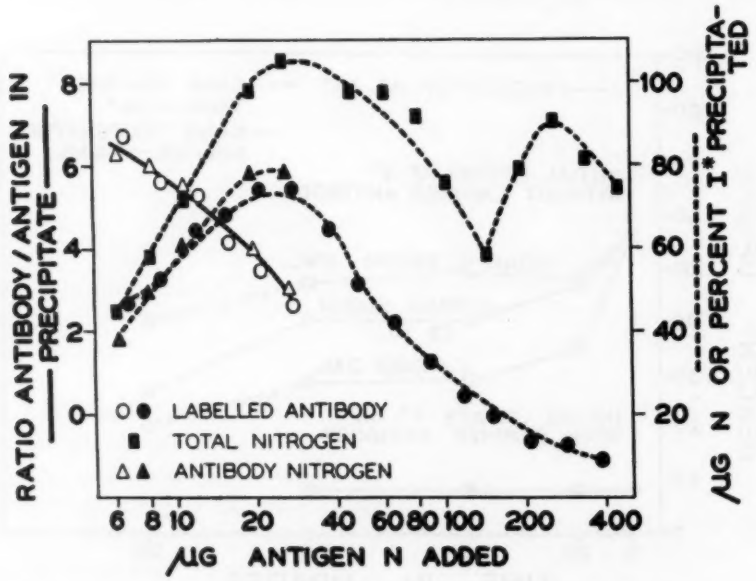

FIG. 5.—I*anti-HGG precipitate fraction and anti-HGG precipitin nitrogen curves made under identical conditions of total antibody concentration (approximately 100 μg antibody N/ml). I*anti-HGG is from an acid eluate.

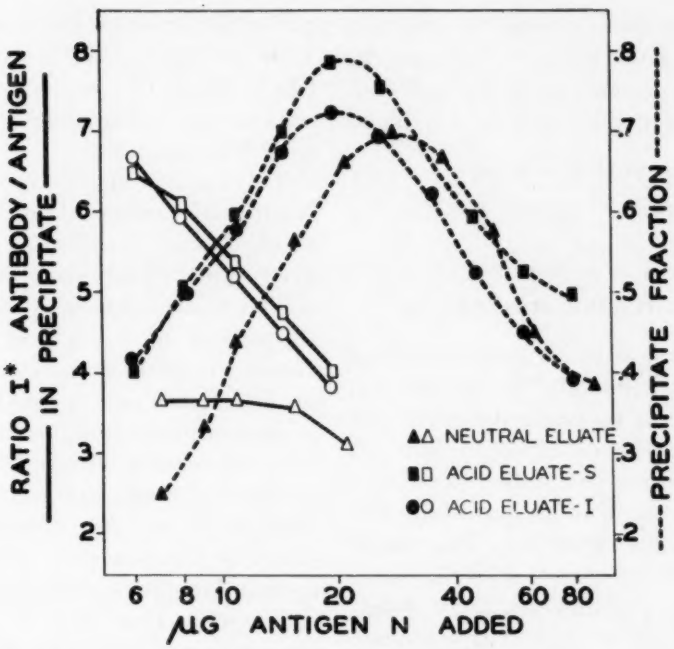

FIG. 6.—I*anti-HGG precipitate fraction curves made under identical conditions of total antibody concentration (approximately 100 µg antibody N/ml). Acid eluate-S is an acid exchange eluate prepared with soluble HGG. Acid eluate-I is an acid exchange eluate prepared with insoluble HGG-cellulose.

tion process, presumably due to the fact that the original absorption was made at approximately the equivalence ratio of the HGG-anti-HGG system.

Although the three curves in figure 6 are roughly comparable in the antigen excess zone, the curve obtained with the neutral eluate shows a significant decrease in precipitation in the region of antibody excess. This decreased precipitation is best seen in a plot of the ratio of labelled antibody to antigen in the precipitate (open symbols in fig. 5 and 6). While the curves obtained with the acid eluates and the antibody N curve showed a ratio of antibody to antigen in the region of antibody excess approximately double that found at equivalence, the neutral eluate demonstrated little change in this ratio. The relative decrease in the precipitation of the labelled antibody under conditions where there is a competition for antigen by a large excess of carrier antibody is interpreted as indicating a lower average avidity of the labelled antibody as compared to the carrier. The low avidity of the labelled antibody in the neutral eluate is probably due to the fact that this eluate contains only that fraction of the labelled antibody reversible at a neutral pH.

I. Stability of labelled antibody in vivo

An amount of each labelled antibody solution containing between 300,000 and 500,000 counts per minute was injected intravenously into a rabbit of known weight. Approximately 2 ml of blood were let into a weighed tube 15 minutes after injection and at daily intervals for 1 week. The tubes were capped immediately, and at the completion of the week all the tubes were weighed and counted along with a measured aliquot of the solution in-

jected. From these determinations may be calculated the percent of the per kg injected dose remaining in 1 g of blood.

Percent of per kg dose in 1 g blood

$$= \frac{\text{counts in sample}}{\text{weight of sample in g}}$$

$$\times \frac{\text{weight of animal in kg}}{\text{total counts injected}}$$

For an animal with a blood volume of 65 ml (approximately 70 g) per kg, 1.4% of the per kg injected dose should be found in 1 g of blood initially. A lower 15 minute value indicates either a large blood volume or an immediate loss of label from the circulation. The results were graphed and the slope of the curve between the 2nd and 7th days determined in terms of half disappearance time. The results from the neutral and acid eluates are given in table 4. No significant differences between the various eluates is apparent, with the exception of an eluate prepared using dilute HCl instead of citric acid for acidification and without normal rabbit serum. In this case there was a rapid drop of radioactivity from the blood during the first 24 hours, followed by a moderately increased half-life between the 2nd and 7th days postinjection.

SUMMARY

Labelled anti-human γ globulin of high isotopic specificity has been prepared by specific absorption or precipitation of the labelled antibody followed by: (1) elution at a neutral pH with carrier exchange, (2) elution at an acid pH with carrier exchange or (3) elution with acid alone. The first two methods can be applied to both soluble and insoluble antigens, the third method to insoluble antigens only.

The various eluates have been analyzed and compared with respect to yield, specific activity, avidity, and in vivo stability (summary in table 1).

The neutral exchange eluate has a theoretical advantage of having received the gentlest treatment, although no deleterious effects could be detected in respect to the functions tested following acidification with citric acid in the presence of normal rabbit serum. A disadvantage of the neutral exchange eluate is the fact that it was found to contain labelled antibody of lower average avidity than that in the original antiserum or in the acid eluates. In addition, the acid exchange eluate had the advantage of highest yield, and the elution with acid alone gave the highest specific activity.

With soluble antigens such as human γ globulin, labelled antibody can be most easily separated by precipitation with the soluble antigen and elution at pH 3.2 with excess carrier. For some in vitro studies, however, where labelled antibody of high specific activity is required, elution of the antibody from an insoluble antigen with acid alone has definite advantages. This was particularly true in the studies reported here concerning the rate, extent, and reversibility of the primary reaction between antigen and antibody under varying conditions of acid, temperature, and antigen concentration.

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